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(54) Title: SPECIFIC ANTIBODIES AGAINST MAMMARY TUMOR–ASSOCIATED MUCIN, METHOD FOR PRODUCTION AND USE

(57) Abstract

An immunologically active polypeptide which specifically binds to the carbohydrate structure of the MUC1 tandem repeat from carcinoma cells, wherein a) the quotient between the affinity of the said polypeptide for a 200 to 440 kDa glycoprotein fraction from tumor cell–containing ascites of breast cancer patients and for native MUC1 antigen (400 to 440 kDa) from normal cells is 100:1 or more, b) the polypeptide does not bind to nonglycosylated MUC1 antigen, and c) the binding of the polypeptide to the said 200 to 440 kDa glycoprotein fraction changes by 10 % or less if the glycoprotein fraction was treated with neuraminidase to cleave N–terminal neuraminic acids, or with formalin, is specific for MUC1 and is useful in the diagnosis and therapy of breast cancer.

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Specific antibodies against mammary tumor-associated mucin, method for production and use

The invention relates to novel specific antibodies against the mammary tumor-associated mucin antigen MUC1, a method for its production and its use in diagnostics and therapeutics.

The MUC1 tumor-associated antigens (polymorphic epithelial mucin, PEM) are high molecular weight glycoproteins (> 200 kDa) with multiple oligosaccharide side chains in O-linkage to serine and threonine residues. They are encoded by a hypervariable gene locus on chromosome 1 (region 21q). MUC1 is a transmembrane glycoprotein of the luminal surface of ductal epithelial cells which extends more than 200-500 nm from the cell surface. It contains a unique extracellular domain consisting mainly of tandem repeats of 20 amino acids. The number of tandem repeats (range 20-120, mean 60) in the MUC1 gene varies significantly (VNTR, variable number of tandem repeats) as a result of genetic polymorphism. In each tandem repeat up to 4 serine/threonine residues are used for glycosylation. The short sugar side chain starts with N-acetylgalactosamine and galactose (core 1 structure). In normal epithelial cells the core 1 structure is then extended by the addition of N-acetylglucosamine, fucose and sialic acid.

The physiological function of the mucins has not yet been fully clarified. It is very probable that in many organs they protect epithelial cells against changes in pH and against mechanical and microbial damage. Mucins are secreted in larger amounts by differentiated breast epithelium in milk, and also in other organs with secretorial epithelia.

In tumor cells MUC1 expression shows a number of significant changes. These include

a) loss of the polarized and restricted luminal expression;

b) up-regulation and secretion;

c) aberrant glycosylation resulting in the appearance of novel carbohydrate epitopes and unmasking of cryptic peptide epitopes.

Mucins therefore have a considerable impact as markers for diagnostic and therapeutic approaches. Underglycosylated MUC1 is highly expressed on breast, ovarian, pancreatic and other cancer cells and can be recognized by HLA-restricted as well as non-MHC-restricted T cells. Several groups have demonstrated that the APDTR epitope of the MUC1 VNTR is the

target of cytotoxic T lymphocytes isolated from patients with breast and other cancers (overview by Finn et al., 1995). In addition, the existence of a B cell immune response was documented in patients with ovarian carcinoma and breast cancer (Rughetti, A., et al., Cancer Res. 53 (1993) 2457-2461). Therefore, MUC1 is a target for cellular immunotherapy, e.g., with a vaccine of MUC1-transduced dendritic cells (DCs) generated ex vivo from CD34 enriched peripheral blood cells from breast cancer patients undergoing high-dose chemotherapy and PBSCT (peripheral blood stem cell therapy). Other possibilities may be the active immunization with synthetic MUC1 peptides, underglycosylated MUC1 or MUC1-DNA. Another possibility is the passive immunization using murine, humanized or human mabs with specificity for MUC1, preferably antibodies reactive with glycone or peptide epitopes of underglycosylated mucin produced by carcinoma cells.
At the ISOBM TD-4 workshop in San Diego, November 17-23, 1996 (Price, M.R., et al., Summary Report on the ISOBM TD-4 Workshop: Analysis of 56 Monoclonal Antibodies against MUC1 Mucin, Tumor Biol. 19, suppl. 1(1998) 1-20); S. Karger AG, Basel, CH), 56 monoclonal antibodies against the MUC1 mucin were analyzed. 16 research groups participated in this workshop. The majority of antibodies (34/56) defined epitopes located within the 20 amino acid tandem repeat sequence of the nonglycosylated MUC1 mucin protein core. Most of these Mabs were reactive with the hydrophilic determinant (PDTRPA) of the MUC1 protein core.

Of the remaining 22 antibodies there was evidence of the involvement of carbohydrate residues in the epitopes for 16 antibodies (Table 1). There was no obvious relationship between the type of immunogen and the specificity of each antibody. Synthetic peptides and glycopeptides were analyzed for their reactivity with each antibody either by assay of direct binding or by determining of the capacity of synthetic ligands to inhibit antibody binding interactions. Carbohydrate epitopes were less easily characterized than the peptide epitopes (Galania, O.E., et al., Tumor Biol. 19, suppl. 1 (1998) 79-87). Carbohydrate residues were involved in many epitopes by regulating epitope accessibility or masking determinants or by stabilizing preferred conformations of peptide epitopes within the MUC1 protein core. Therefore, the characterization of carbohydrate epitopes was more of a problem than the protein core epitopes.

The potential number of permutations for linear and branched isomers of small oligosaccharides can run into ten of thousands (Laine, R.A., Glycobiology 4 (1994) 1-9). The findings from this workshop highlight some of the complexity in assigning/defining carbohydrate epitopes for antibodies against MUC1 (Lloyd, K.O., Tumor Biol. 19, suppl. 1

(1998) 118-121). Eight of these mabs did not react with soluble and tumor-associated MUC1 of ZR75 breast carcinoma cells. From the remaining 8 mabs 3 were reactive with synthetic MUC1 peptides. Only four mabs were nonreactive to the monomeric MUC1 tandem repeat peptide but had strong binding to the lactation-associated MUC1 secreted into milk. A very restricted reactivity was exerted by clone FH6, however this mab with sialosyl-Le^X glycolipid specificity (Fukushi, J., et al., J. Biol. Chem. 259 (1984) 10511-10517) is weakly reactive only with membrane spots and the Golgi complex in T-47D cells. The binding of FH6 to MUC1 is strongly affected by neuraminidase treatment.

The anti-MUC1-antibody 12H12 (Bastert, G., et al., in Rygaard, Brünner eds., Immuno-deficient animals in biomedical research, Basel, Karger, 1987, 224-227) reacts with a tumor-associated antigen known as TAG12 that is expressted by 96% of all breast cancers. The antigen can be found in high concentrations in the cytoplasm and cell membrane of breast cancer tissue and metastases and is secreted in tumor cells.

This antibody could be used for immunoscintigraphy of human mammary carcinoma xenografts as described by Brümmendorf, T.H., et al., Cancer Research 54 (1994) 4162-4168 and Nucl. Med. 34 (1995) 197-202. However, the extent of specificity of these antibodies too is not sufficiently high for rendering them suitable for routine diagnostics and therapeutic application.

In addition 12H12 was used together with the MUC1 peptide specific Mab 2E11 (Diel, J.J., Natl. Cancer Inst. 88 (1996) 1652-1658) for the analysis of micrometastatic tumor cells in bone marrow.

Bone metastases are common in breast cancer, large autopsy studies giving their frequency at 47-85% (Weiss, L., and Gilbert, H.A., Bone Metastasis, Boston, GK Hall, 1981). The goal of any adjuvant therapy in breast cancer is the destruction of the smallest subclinical tumor sites, yet there is still no routine diagnostic method that can detect such "micrometastases". Since breast cancer tends to metastasize to bone in particular, numerous attempts have been made to find malignant cells in bone marrow. Bone marrow as a target organ of metastasis is easily accessible and can be aspirated with relatively little danger and pain from the patient. Detecting "cells at the wrong place" can provide information about early phase of the metastatic cascade and possibly define the metastatic potency of the primary tumor.

It is known that analysis of tumor cell contamination in the bone marrow of breast cancer patients is a powerful predictor of patient outcome (Diehl, I.J., J. Natl. Cancer Inst. 88 (1996)

1652-1658). Enrichment of rare tumor cells with anti-MUC1 antibodies immobilized on magnetic beads results in a dramatically improved sensitivity of cytochemical tumor cell detection (Kaul, S., et al., Abstract 51, Kongreß der Deutschen Gesellschaft für Gynäkologie und Geburtshilfe, Dresden, 1.-5.10.96).

However, the value of such methods is limited by the specificity of the antibodies used. A large number of antibodies against different epitopes of human neoblastic breast tissue have been investigated previously. Many of them were directed to a carcinoembryonic antigen (DeLand, F.H., et al., J. Nucl. Med. 20 (1979) 1243-1250), TAG72 (Lamki, M., et al., J. Nucl. Med. 32 (1991) 1326-1332), cell surface antigens, such as the MAbs B6.2 (Colcher, D., et al., Proc. Natl. Acad. Sci. USA 78 (1981) 3199-3203), anti-MME (Wilbanks, T., Cancer 48 (1981) 1768-1775), or various others. The majority of the MAbs used for breast cancer have been shown to recognize epitopes on mucin molecules (Taylor-Papadimitriou, J., Int. J. Cancer 49 (1991) 1-5). However, a highly specific and sensitive monoclonal antibody for diagnostic and therapeutic purposes has not yet been found (Merino, M.J., et al., Nucl. Med. Biol. 18 (1991) 437-443).

However, none of the known anti-MUC1-antibodies are sufficiently highly specific so as to be suitable for broad use in diagnostics and therapy.

It is the object of the invention to provide new highly-specific antibodies, a method for their production, and their diagnostic and therapeutic use.

Accordingly, a subject-matter of the invention is an immunologically active polypeptide which specifically binds to the carbohydrate structure of the 20 amino acid tandem repeat of MUC1 from tumor (carcinoma) cells, wherein

a) the quotient between the affinities of said polypeptide for a 200 to 440 kDa underglycosylated glycoprotein fraction (analyzed by SDS-PAGE) from tumor cell-containing ascites from breast cancer patients and for native MUC1 antigen (400 - 440 kDa) from human milk is 100 : 1 or higher;

b) the polypeptide does not bind to nonglycosylated MUC1 antigen, and

c) the binding of the polypeptide to the said 200 to 440 kDa glycoprotein fraction (analyzed by SDS-PAGE) changes by 10% or less if the glycoprotein fraction was treated with neuraminidase to cleave N-terminal neuraminic acids, or was treated with formalin, preferably with 3.5% formalin, in phosphate buffered saline (PBS) for 15 to 30 minutes.

Such polypeptides with antibody activity and characteristics have surprisingly been found to be highly specific for MUC1 presented by tumor cells. Therefore, such antibodies are useful tools for tumor diagnosis and therapy.

It has surprisingly been observed that such antibodies are reactive with carbohydrate epitopes of underglycosylated MUC1, which are overexpressed and secreted by breast carcinoma and other carcinomas. Such antibodies can be selected by a new immunization protocol. Ascites fluids from patients with advanced breast cancer were used as antigen source. The MUC1 antigen fractions in these fluids were enriched by a two-step-procedure using lectin affinity chromatography, and size exclusion chromatography. An essential aspect of the present invention is such a procedure which favors the selection of antibodies according to the invention with strong reactivity for secreted MUC1 but minimal reactivity for normal milk MUC1, deglycosylated milk mucin and/or normal urinary mucin.

It is essential for the characterization of the antibodies according to the invention that a MUC1-containing glycoprotein fraction is used which can be isolated via lectin affinity chromatography (preferably, WGA, bound to agarose) from a mixture of ascites from different tumor patients, which contains secreted proteins. In a second step, the native glycoprotein peak fraction of 1,000 kDa (measured by FPLC, corresponding to 200-440 kDa in the SDS gel) is separated, whereby, amongst others, the immunoglobulins are removed by FPLC gel filtration. Immunization according to the state of the art is carried out with the so purified protein fraction and hybridoma clones are screened, said hybridoma clones having the following properties:

- minimal reaction with antigen isolated from human milk and/or urine;
- maximal reaction with the WGA fraction;
- no binding to a nonglycosylated (synthetic) peptide as is, for example, described in International Application No. WO 90/05142;
- binding to the WGA fraction even after the N-terminal neuraminic acids were separated off from the WGA fraction by neuraminidase or formalin;
- binding to the carbohydrate structure of the 20 amino acid tandem repeat of MUC1 from tumor cells.

„Specific binding“ in the sense of the invention means that the antibody according to the invention does not bind to the peptidic structure of MUC1 and binds slightly to the carbohydrate

structure of MUC1 from human milk, while binding strongly to the carbohydrate structure of MUC1 from tumor cells.

The WGA fraction from ascites (underglycosylated MUC1) is characterized essentially by the fact that it contains very little of the native fully glycosylated antigen (400 to 440 kDa) but exhibits in the SDS gel strong bands between 200 and 220 kDa (cf. Fig. 1). This difference in molecular weight is due to hypoglycosylation (underglycosylation). "Underglycosylation", on the molecular basis, means that at one or more of the tandem repeats one or more of the glycosylation sites are nonglycosylated or are not occupied with the sugar residues as in the native molecule.

Since the average number of repeats contained in MUC1 is about 60, whereby a maximum of three sugar chains may be located at each repeat, fully glycosylated MUC1 contains about 150 to 200 sugar side chains. The average number of sugar residues per side chain is about 10. In underglycosylated MUC1, each underglycosylated glycosylation site is a potential binding site for the antibodies of the invention. The less the glycosylation of the sugar side chains, the higher the number of antibodies that are capable of binding. Already when at least 10, preferably at least 20, sugar side chains are missing or underglycosylated, an appreciable binding of the antibody according to the invention to MUC1 is to be observed.
Another subject-matter of the invention is a method for the production of an immunologically active polypeptide which specifically binds to a hypoglycosylated MUC1 fraction from tumor cells with a molecular weight of 200 to 400 kDa, preferably 200 to 220 kD (as analyzed by SDS-PAGE), wherein the glycoproteins are isolated from tumor cell-containing ascites of breast cancer patients by lectin affinity chromatography, said glycoproteins are optionally separated from the Ig proteins, and the fraction so obtained is used for the immunization of animals, antiserum is obtained, and the polypeptide is isolated therefrom, said polypeptide being characterized in that

a) the quotient between the affinities of the said polypeptide for a 200 to 400 kDa underglycosylated glycoprotein fraction (analyzed by SDS-PAGE) from tumor cell-containing ascites of breast cancer patients and of native MUC1 antigen (400 - 440 kDa) from human milk is 100 : 1 or more,
b) the polypeptide does not bind to nonglycosylated MUC1 antigen, and

c) the binding of the polypeptide to the said 200 - 400 kDa glycoprotein fraction changes by 10% or less if the glycoprotein fraction was treated with neuraminidase to cleave N-terminal neuraminic acids, or with formalin.

As used herein, the term "immunologically active polypeptide or antibody" refers to a protein consisting of one or more polypeptides substantially encoded by antibody genes. The recognized antibody genes include the different constant region genes as well as the myriad antibody variable region genes. Antibodies may exist of a variety of forms, including, for example, Fv, Fab, and $F(ab)_2$ as well as single chains (e.g., Houston et al., PNAS USA 85 (1988) 5879-5883 and, in general, Hood et al., Immunology, Benjamin N.Y., 2nd edition (1984) and Hunkapiller and Hood, Nature 323 (1986) 15-16. Preferred antibodies according to the invention are monoclonal antibodies and fragments thereof.

The relative affinity of anti-MUC1 antibodies was determined by the analysis of the K_{50} value, which is the antibody concentration at which half-maximal binding to MUC1 is achieved (Karanikas, V., et al., Tumor Biol. 19, suppl. 1 (1998) 71-78). In the ELISA binding assay affinity purified MUC1 antigen was coated in microtiter wells. Using a molecular weight of 160,000 for IgG the K_{50} value for 7F11 was 10^{-10} M with tumor associated antigen from ascites and T-47D breast cancer cells.

The murine monoclonal antibodies 1E4 and 7F11 were selected after immunization with MUC1 antigen fractions purified from ascites of patients with advanced breast cancer. The antibodies are reactive with carbohydrate epitopes of underglycosylated MUC1 which is overexpressed and secreted by breast and other carcinomas. The antibodies are useful tools for tumor diagnosis and therapy.

The antibodies according to the invention, preferably the antibodies 7F11 and 1E4 are clearly distinct from all 16 carbohydrate specific mabs submitted to the ISOBM TD-4 international workshop on monoclonal antibodies against MUC1 (1996). Both mabs are reactive with high affinity ($K_{50} = 10^{-10}$ M) with tumor-associated MUC1 from different sources (primary tumor, metastatic cells, secreted antigen). In contrast to the TD-4 mabs (table 1) 7F11 and 1E4 are unreactive with normal, fully glycosylated milk MUC1. They are distinct from clone FH6 by a completely different staining pattern and by the fact that the FH6 immune reactivity is impaired by desialylation of MUC1 (treatment with neuraminidase or formalin).

The antibodies can be used as whole monoclonal antibodies, fragments thereof (e.g. Fv, $(Fv)_2$, Fab, Fab', $F(ab)_2$), chimeric, humanized or human antibodies as long as they are binding to MUC1 in a suitable manner. Short-chain antibody fragments containing only the CDR regions or parts thereof conferring the specific binding to MUC1 are also suitable, especially if the antibody is a labelled one. Antibodies of the IgG1 isotype are preferred.
As to production of monoclonal antibodies see, for example, E. Harlow and D. Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Press (1988); Bessler et al., Immunobiol. 170 (1985) 239-244; Jung et al., Angewandte Chemie 97 (1985) 883 or Cianfriglia et al., Hybridoma Vol. 2(1993) 451-457.

The use of the antibodies according to the invention for diagnostic use thereby takes place in the known manner by means of an immunological process of determination. Processes of this type are well-known and do not need to be further explained here. The antibodies obtained according to the present invention can be used as unlabelled and/or immobilized receptors.

Immobilized receptors can preferably be used for a method for the removal of tumor cells or secreted MUC1 antigen from a patient's body material, wherein the body material is brought into contact with an immobilized antibody as claimed in claim 1 or 2, he tumor cells or said secreted MUC1 antigen are bound to the immobilized antibody, and the tumor cells or said secreted MUC1 antigen so bound to the solid phase are separated along with the solid phase from the body material.

In each case of such immunological method of diagnosis, there is evaluated a signal change following the binding of at least one antibody according to the invention, to which is bound a detectable label.

Since the monoclonal antibodies obtained by the process according to the present invention bind to the surface of tumor cells, they can be used for in vivo treatment in humans. Thus, the present invention also provides a pharmaceutical composition which comprises one or more antibodies according to the present invention, optionally together with conventional pharmaceutical carrier, adjuvant, filling or additive materials. The administration of a

medicament according to the present invention is useful for the treatment of micrometastases, especially in the treatment of breast cancer.

A suitable dosage of the antibody according to the present invention for the therapeutic treatment is about 2 µg to 20 mg, preferably 50 µg to 20 mg /kg body weight, whereby this dosage possibly is to be repeatedly administered.

For the treatment of patients with breast carcinoma with the antibodies according to the invention it is preferred to carry out, after the surgery, an established adjuvant chemotherapy (before menopause) or hormone therapy (after menopause) in lymph-node-positive patients (about 45%). In a further step, the presence of tumor cells in the bone marrow is diagnosed. This is done preferably with the use of the antibodies according to the invention, but can be accomplished also with other tests recognizing usual tumor markers (cytokeratins, etc.). If the bone marrow reaction is positive, preferably 6 to 8 immunizations are performed at a 1 to 4-week-interval using the antibody according to the invention. The amount of antibody used is in the range of 0.2 to 50 mg per immunization. This antibody therapy is carried out during adjuvant hormone therapy or after chemotherapy. In the case of hormone therapy, it may be carried out preferably in a parallel manner (no immunosuppression in the latter case).
The development of antibodies against the anti-MUC1 antibodies according to the invention is being monitored during the phase of immunization with the antibodies according to the invention or by established tests for human anti-mouse antibodies (HAMA). The desired therapy level is achieved when the antibody titer has been stable for about three to four months. Another determination of the tumor cells in the bone marrow is performed three months after the last immunization. At least 6×10^6 bone marrow cells are analyzed for tumor cell contamination. It has been found that when using the antibodies according to the invention, 17 out of 18 patients exhibit negative reactions in the assay for tumor cells in bone marrow, which implies that the tumor cells in the bone marrow have obviously been destroyed as a result of the therapeutic application of the antibodies according to the invention. Surprisingly, the antibodies of the invention exhibit, in addition, an effector function, whereby, after binding to the tumor cells, the tumor cells are recognized as foreign cells and are presumably eliminated by the activation of the complement, anti-idiotypic antibodies, activated NK-cells or T-cells or a combination of the latter.

It has been shown further that the antibodies of the invention bind also to secreted MUC1 in body fluids such as serum, thereby having a therapeutic effect by elimination of MUC1-induced

immunosuppression, by humoral or cellular immune recognition, or as an immunosuppressive agent. This too can be utilized for therapy.

It has been been found further that MUC1 is overexpressed also in tumor cells of the ovary (>80%), the lung (60%), prostate (50%), pancreas (100%), kidney, and colon. The antibodies of the invention can be utilized here too for diagnosis and therapy.
It is specifically preferred to use, for therapeutic purposes, cytotoxic antibodies which impart effector functions (ADCC, CDC) (Brüggemann et al., J. Exp. Med. 166 (1987) 1357-1361).

In another approach, the antibody or part of it is conjugated or translationally fused to a toxin molecule (immunotoxin), thus effecting specific killing of tumor cells (Brinkmann et al., Proc. Natl. Acad. Sci. USA 88 (1991) 8616-8620; Pastan et al., Cancer Res. 51 (1991) 3781-3787; FitzGerald and Pastan, J. Natl. Cancer 81 (1989) 1455-1461) or conjugated to a cytokine or interleukin. In another preferred embodiment of the invention, bispecific antibodies are used for tumor therapy (Bonino et al., BFE 9 (1992) 719-723), which may be constructed by in vitro reassociation of polypeptide chains, by hybrid hybridoma generation or by construction of diabodies (Holliger et al., Proc. Natl. Acad. Sci. USA 90 (1993) 6444-6448; Holliger and Winter, Current Opin. Biotechnol. (1993) 446-449).

With regard to immunotoxins, it is preferred to couple the antibody according to the invention to a toxin, such as, for example, Pseudomonas exotoxin, Diphtheria toxin or other toxins (FitzGerald and Pastan, J. Natl. Cancer 81 (1989) 1455-1461). It is also preferred to couple the antibodies to chemotherapeutics, such as, for instance, doxorubicin, or to radioactively labelled substances which have a cytotoxic effect.

Conjugates of the antibodies according to the invention, in particular of human antibodies, for in vivo imaging, using, for instance, radioactive or fluorescent substances, are also preferred.

Immunotoxins can be produced preferably by either of two principally different methods:

In one method, an antibody or a fragment thereof (normally generated proteolytically, e.g. Fab-fragment) is chemically coupled in vitro to a toxin or toxin fragment. For practical reasons, the antibody part in this type of immunotoxin is either a complete antibody (consisting of two light and two heavy chains) or, more preferably, a Fab-fragment (consisting of one light chain and the VH- and CH1-regions of the heavy chain).

In another method, the immunotoxin is generated by recombinant DNA techniques, which leads in any case to a defined, homogeneous molecule. The size of the antibody part should be as small as possible to obtain a small immunotoxin with good tissue penetration. In this method, the smallest practically available antibody fragment is not the Fab-fragment, but the functional variable domain of an antibody, consisting of the VH-region of the heavy chain and the VL-region of the light chain only. VH- and VL-region (polypeptide chains each of about 100 amino acids) have to form a functional assembly, the variable domain, which confers antigen binding. In the absence of any of the remaining parts of an antibody, VH- and VL-region form very labile complexes only. Therefore, their complex is preferably stabilized by covalent bonds.

One possibility is to fuse on the DNA level VH-region, VL-region (or vice versa) and the toxin part. Upon expression, a single polypeptide chain is formed, wherein VH- and VL-region, being connected by a peptide linker, fold into a stable variable domain, while the toxin is fused e.g. to VL via a second peptide linker (see Brinkmann et al., Proc. Natl. Acad. Sci. USA 89 (1992) 3075-3079). The length of both peptide linkers is variable and may in some instances even be reduced to a single peptide bond. A molecule of this type has been termed a "single chain immunotoxin", analogous to the term "single chain antibody" or scFV, which is used for a single polypeptide chain containing both VH and VL connected by a peptide linker or bond.

Another possibility to stabilize the VH- and VL-assembly is described in Brinkmann et al., Proc. Natl. Acad. Sci. USA 90 (1993) 7538-7542). In this technique, amino acids on VH and VL were defined by computer aided modelling, which are closely adjacent in the VH-VL-complex. The naturally occurring amino acids in these positions were then on the DNA level replaced by a cystein each. To obtain a functional immunotoxin in this case, two separate polypeptide chains are expressed (in separate cells, e.g. prokaryotic cells, e.g. E.coli), one being the VH-region only, the other the VL-region fused by a peptide linker to the toxin part. These two polyeptide chains are mixed under appropriate conditions and thus assemble into a functional immunotoxin, where VH and VL in the variable antibody domain are connected by a disulfide bond between the two cysteins introduced by genetic engineering. The antibody part of this type of immunotoxin has been designated dsFV and the whole molecule consequently as "dsFV-immunotoxin".

There exist, of course, additional possibilities to produce immunotoxins by recombinant DNA techniques, for instance by using the larger Fab-fragment (VH-CH1 non-covalently assembled to VL-CL, while one of them is fused by a peptide linker to the toxin). However, the possibilities

described by Brinkmann et al., Proc. Natl. Acad. Sci. USA 89 (1992) 3075-3079 and Brinkmann et al., Proc. Natl. Acad. Sci. USA 90 (1993) 7538-7542 are to be preferred.

With respect to the toxin part of the immunotoxin, preferred fragments of the Pseudomonas exotoxin (PE) are PE38 and PE40 and derivatives thereof (I. Pastan et al., WO 92/07271, WO 90/12592).

Single chain F_V-chain immunotoxin is preferably produced as a single polypeptide chain in E.coli, using the T7 RNA polymerase expression system. The polypeptide is obtained in an inactive form and has to be activated by in vitro renaturation.

Other methods for the production of peptide bonded single chain immunotoxins are described in WO 88/09344. Single chain antibodies with a peptide linker between the light and the heavy chain are described in WO 88/01649. The production of chimeric antibodies which comprises at least the variable regions of a heavy and light chain whereby one of these chains is linked by a peptide bond to a non-Ig molecule are described in EP-B 0 193 276. The T7 RNA polymerase expression system is described in US Patent Nos. 7,648,971, 4,952,496 and 6,595,016.

Polynucleotides of the invention and recombinantly produced antibodies of the invention may be prepared on the basis of the sequence data according to methods known in the art and described in Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd edition (1989) Cold Spring Harbor, New York, and Berger and Kimmel, Methods in Enzymology, Vol. 152, Guide to Molecular Cloning Techniques (1987), Academic Press Inc., San Diego, CA, which are incorporated herein by reference. Polynucleotides of the invention are preferably formed from synthetic oligonucleotides.

Such recombinant polypeptides can be expressed in eukaryotic or prokaryotic host cells according to standard methods known in the art; preferably mammalian cells, such as lymphocyte cell lines, may be used as host cells. Typically, such polynucleotide constructs encode a complete human antibody heavy chain and/or a complete human antibody light chain having at least the amino acid sequences of the antibodies according to the invention, heavy and/or light chain variable regions respectively. Alternative human constant region sequences (heavy and/or light chain) other than those naturally associated with said antibody chains may be substituted, including human constant region isotypes, such alternative human constant region sequences can be selected by those of skill in the art from various reference sources,

including, but not limited to, those listed in E.A. Kabat et al., Sequences of Proteins of Immunological Interest (1987), National Institute of Health, Bethesda, MD. In one embodiment of the invention, a polynucleotide sequence encoding an antibody light chain comprising a human light chain, constant region with an amino terminal peptide linkage (i.e. an inframe fusion) to a variable region of the light chain of the antibody according to the invention and a corresponding heavy chain are expressed and form heavy/light chain dimers and other antibody types.

In general, prokaryotes can be used for cloning the DNA sequences encoding an antibody chain according to the invention. E.coli is one prokaryotic host particularly useful for cloning the DNA sequences of the present invention. Alternatively, oligonucleotides may be synthesized chemically by a variety of methods, including phosphoramidite synthesis.

The polynucleotide constructs will typically include an expression control sequence operatively linked to the coding sequences, including naturally associated or heterologous promotor regions. Preferably, the expression control sequences will be eukaryotic promotor systems in vectors capable of transforming or transfecting eukaryotic host cells. Once the vector has been incorporated into the appropriate host, the host is maintained under conditions suitable for high level expression of the nucleotide sequences and the collection and purification of the antibodies according to the invention. As eukaryotic host cells, mammalian tissue cell cultures may also be used to produce the polypeptides of the present invention. Mammalian cells are actually preferred, because a number of suitable host cell lines capable of secreting intact heterologous proteins have been developed in the art and include the CHO cell lines, various COS cell lines, HeLa cells, myeloma cell lines, etc.

Typically, the polynucleotide sequences encoding the heavy and/or light chains of the antibody according to the invention are introduced into and expressed in glycosylating cells which glycosylate the antibody. As used herein "glycosylating cell" is a cell capable of glycosylating proteins, particularly eukaryotic cells capable of adding an N-linked "core oligosaccharide" containing at least one mannose residue and/or capable of adding an O-linked sugar to at least one glycosylation site sequence in at least one polypeptide expressed in said cell, particularly a secreted protein. Thus, a glycosylating cell contains at least one enzymatic activity that catalyzes the attachment of a sugar residue to a glycosylating site sequence in a protein or polypeptide and the cell actually glycosylates at least one expressed polypeptide. For example,

but not for limitation, mammalian cells are typically glycosylating cells. Other eukaryotic cells such as insect cells and yeast may be glycosylating cells.

Once expressed, antibodies according to the invention can be purified according to standard procedures of the art, including HPLC purification, fraction column chromatography, gel electrophoresis, and the like (see generally, R. Scopes, Protein Purification, Springer Verlag, N.Y. (1982)).

The therapeutic compounds of this invention may be administered parenterally, such as intravascularly, intraperitoneally, subcutaneously, intramuscularily, using forms known in the pharmaceutical art. The active drug components of the present invention are used in liquid, powdered or lyophilized form and may be combined with a suitable diluent or carrier, such as water, a saline, aqueous dextrose, aqueous buffer, and the like. Preservatives may also be added.

Regardless of the route of administration selected, the compounds of the present invention are formulated into pharmaceutically acceptable dosage forms by conventional methods known to those skilled in the art. The compounds may also be formulated using pharmacologically acceptable acid or base addition salts. Moreover, the compounds or their salts may be used in a suitable hydrated form.

Regardless of the route of administration selected, a non-toxic but therapeutically effective quantity of one or more compounds of this invention is employed in any treatment. The dosage regimen for treating is selected in accordance with a variety of factors including the type, age, weight, sex and medical condition of the patient, type of tumor, the route of administration and the particular compound employed in the treatment. A physician of ordinary skill can readily determine and prescribe the effective amount of the drug required regarding known antibody therapy approaches. In so proceeding, the physician could employ relatively low doses at first, and subsequently, increased dose until a maximum response is obtained.

Pharmaceutical compositions comprising an antibody of the present invention are useful for topical or parenteral administration, i.e. subcutaneously, intramuscularly, intravenously or transdermally. The compositions for parenteral administration will commonly comprise a solution of said antibody dissolved in an acceptable carrier, preferably in an aqueous carrier. A variety of aqueous carriers can be used, e.g. water, buffered water, 0.4% saline, 0.3% glycin,

and the like. The solutions are sterile and generally free of particulate matter. The compositions may be sterilized by conventional well-known techniques. The compositions may contain pharmaceutically acceptable auxiliary substances, such as are required to approximate physiological conditions, such as pH adjusting and buffer agents, toxicity adjusting agents, and the like, for example sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate, etc. The concentrations of the antibodies according to the invention in these formulations can be varied widely, e.g. from less than about 0.01%, usually at least about 0.1%, to as much as 5% by weight, and will be selected primarily based on fluid volumes, viscosity, etc. or in accordance with the particular mode of administration selected.

Thus, a typical pharmaceutical composition for intramuscular injection could be made up to contain 1 ml sterile buffered water and about 1 to 50 mg of antibody according to the invention.

The antibodies according to the invention can be lyophilized for storage and reconstituted in a suitable carrier prior to use. Conventional lyophilization and reconstitution techniques can be employed. It will be appreciated by those skilled in the art that lyophilization and reconstitution can lead to varying degrees of biological activity loss and that use levels may have to be adjusted to compensate.

The safety, dosage and therapeutic potency of anti-MUC1 antibodies according to the invention was evaluated in clinical case studies with 55 breast cancer patients. Patients with minimal residual disease (n=47) and metastatic disease (n=8) entered the study. All patients had proven tumor cell contamination of the bone marrow at the time of the primary operation, after standard adjuvant therapy and before therapy with the antibody 7F11, respectively. 18 patients received multiple infusions of 50 mg of native 7F11 every 4 weeks. One patient with advanced metastatic breast carcinoma received the 50 mg dose 4 times at weekly intervals. A second group of 14 patients was treated with a 20 mg 7F11 dose, while a third group of 22 patients was under a 2.5 mg treatment regime. In general, the antibody infusions were well tolerated. Only 6 patients treated by a fast (two hours) infusion of 50mg 7F11 developed immediate mild allergic reactions. These side effects could be circumvented by prolonging the infusion time of the 50mg dose to 8 hours. Systemic side effects were not encountered. No adverse side effects were seen in the 20 mg and 2.5 mg treatment groups which had infusion times of 4 and 1 hours, respectively. Immunizations were repeated until stable titers of human anti-mouse antibodies (HAMA) in the range of 1 - 100 µg/ml and anti-7F11 antibodies (ab^2) were reached. Three months later bone marrow was analysed for detection and quantification of

micrometastatic cells. So far, bone marrow of 24 patients was reexamined during and after 7F11 therapy, while the other patients are still under therapy.
Eighteen patients of the M0-group were found to be free of contaminating tumor cells in bone marrow, while in one patient an increase from 3 to 7 tumor cells per 10^6 bone marrow cells was recorded. During follow up all immunized patients remained tumor free. In the meantime seven of these patients received a prophylactic booster immunization as maintenance treatment resulting in dramatic increases of HAMA and anti-7F11 titers. These booster immunizations were tolerated without adverse events and demonstrate that a maintenance treatment with murine 7F11 is feasible despite elevated HAMA and anti-7F11 titers.

In the group of patients with clinically documented local disease or metastasis before 7F11 therapy, elimination of tumor cells in bone marrow was seen in two cases, a reduction was recorded in two patients and an increase of tumor cells was seen in a another patient. Three patients are still under therapy.

One patient with advanced breast cancer received 50 mg 7F11 infusions at weekly intervals. 24 hours after the first treatment serum, bone marrrow and ascitic fluids were analysed for 7F11 biodistrubution. Serum 7F11 levels of 10µg/ml resulted in concentrations of 8 µg/ml in bone marrow and 6 µg/ml in ascites, respectively. Under these conditions tumor cells in bone marrow (>100/10^6 normal bone marrow cells) and in ascites (10 cells/ml) revealed uniform and strong 7F11 labeling of cell membrane and cytoplasm.

Overall, the clinical study shows the safety, tolerance, pharmacokinetics of multiple doses of anti-MUC1 antibodies according to the invention in breast cancer patients with minimal residual disease. Micrometastatic tumor cells in bone marrow (mean 3.3 tumor cells per 10^6 normal bone marrow cells) were eliminated in eighteen patients of the M0 group and in two patients with locoregional disease. No disease progression was seen in these patients during follow up. The study shows, that treatment of breast cancer patients with unconjugated antibodies according to the invention can eliminate tumor cells in the bone marrow either by direct cytotoxicity, passive effector mechanisms or by idiotypic network responses. Moreover, monitoring micrometastatic cells in bone marrow is presented as an extremely sensitive surrogate marker to monitor the efficacy of adjuvant tumor therapies.

Induction of immunity against tumor associated antigens (TAA) like GA733-2(CO17-1A) might eliminate tumor cells or delay recurrence of the disease. Studies in patients with colon cancer using native mAb 17-1A infused repeatedly at high dosage (500 mg) decreased the mortality rate to 30% (Riethmüller, G., Lancet 343 (1994) 1177-1183). Recent results from Fagerberg, J., et al., Cancer Immunol. Immunother. 38 (1994) 149-159) in a group of patients with advanced colon cancer treated with 17-1A show that a) patients with significant ab2 response had longer survival times, b) higher total doses of the native mAb in the range of two gramms administered at short intervals induced lower concentrations of human ab2 and c) recombinant humanized 17-1A induced significantly lower titers of anti-idiotypic antibodies.

Breast mucins are highly glycosylated proteins coded by the gene MUC1 on chromosome 1q. The antigen is overexpressed and aberrantly glycosylated in breast and ovarian carcinomas. Tumor-associated mucins are found in cytoplasm, cell membranes and secretory components.

These cell membrane and secreted TAA can induce humoral (Rughetti, A., et al., Cancer Res. 53 (1993) 2457-2461) and cellular immunity in breast cancer patients (Jerome, H.R., J. Immunol. 151 (1993) 1654-1662). Mabs with MUC1 specificity therefore are the candidates of choice for immuntherapeutic interventions (overview cf. Apostolopoulos, V., and McKenzie, F.C., Critical Rev. in Immunology 14 (1994) 293-309).

Our group has developed a large number of monoclonal antibodies (mAbs) against different epitopes of the human breast mucin (BM). MAb 7F11, specific for multiple carbohydrate/peptide binding sites of the tandem repeat region of the human breast mucin MUC1 is reactive with over 96% of breast carcinomas.

The 7F11 staining pattern in solid tumors and metastases is homogeneous with usually over 80% of stained tumor cells. Therefore 7F11 can be used for the analysis of tumor cells in bone marrow and for immunoscintigraphy.

Histological as well as flow cytometry analysis has documented high tumor selectivity and minimal cross reactivity with normal cells of different tissues for the Mab 7F11. For this reason the mAb was selected for the immunotherapeutic treatment of breast cancer patients with minimal residual disease. The present immunization protocol was designed for

a) idiotypic network activation;

b) B-cell activation;

c) T-cell activation, or

d) antibody-dependent cellular cytotoxicity (ADCC).

7F11 therefore was administered at rather low doses of 2.5, 20 and 50 mg at 4 weeks intervals. Toxic side-effects other than allergic reactions were not encountered in a group of 55 patients. In particular, no anaphylactic reactions were seen. Three patients received a total dose of 450 mg 7F11.

All patients in the study group developed stable and long lasting titers of HAMA and anti-7F11 antibodies after 3 to 10 immunizations, one patient developed stable titers after one immunization already (mean 5.6). The number of immunizations therefore suitably is 1-10, preferably 8-9, and most preferably, 6-8 on a weekly or monthly basis depending on the individual patient and the patient's ability to develop stable and long-lasting antibody titers. Maximal HAMA-titers were in the range of 10 to 100 µg/ml with a mean serum half live time of 5 months after the last immunization. In four patients (patients 1,2, 4 and 5, Table 1) between 25 - 40 % of the anti-idiotypic response is directed toward the antigen binding site and therefore of ab2ß-type.

Patients were reexamined for tumor cell contamination in the bone marrow three months after the last immunization. From every patient bone marrow cells were analysed on at least 6 cytospins by immunocytology and automated picture analysis. 18 patients of the M0 group and two patients with local disease were negative for micrometastatic tumor cells. Only one patient of the M0 group had an increase of tumor cells in bone marrow. These results clearly show that passive immunotherapy with 7F11 can eliminate individual micrometastatic tumor cells in bone marrow in the majority of breast cancer patients in the adjuvant situation.
Unconjugated murine mAbs 7F11 may influence the human immune system by different ways. The direct mechanisms depend on labeling of tumor cells and are triggered by antibody-dependent cellular cytotoxicity, complement-dependent cytotoxicity and apoptosis. Indirect mechanisms may operate via the immune network. Anti-idiotypic antibodies (ab2ß) binding to the hypervariable region of the therapeutic mouse antibody induce corresponding T-cells (T2) and trigger the development of anti-anti-idiotypic antibodies (ab3) und T-cell (T3) clones. Human ab3 antibodies recognizing ab2ß and the nominal antigen epitope of the human breast mucin definded by the therapeutic mouse antibody may be responsible for the favorable outcome of immunized patients.

In conclusion, the 7F11 therapy resulted in dramatic reduction or eradication of micrometastatic breast tumor cells in bone marrow of breast cancer patients in the adjuvant situation.

The cell lines DSM ACC 2329 (1) and DSM ACC 2328 (2) mentioned in the present invention which secrete the antibodies 7F11 (1) and 1E4 (2) were deposited by Boehringer Mannheim GmbH with Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM), Mascheroder Weg 1b, D-38124 Braunschweig, Germany, on October 31, 1997.

The following tables, examples, references, the sequence listing and the drawings are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

List of abbreviations:

HAMA	= human anti-mouse antibody
PBS	= phosphate buffered saline
APAAP technique	= alkaline phosphatase anti-alkaline phosphatase complex
TCD	= number of tumor cells
TAA	= tumor-associated antigens
PUM	= polymorphic urinary mucin-like glycoprotein
EIA	= enzyme immunoassay
PMSF	= phenylmethylsulfonylfluoride
WGA	= wheat germ agglutinin
BSA	= bovine serum albumin
PP	= phosphate buffer
TFMSA	= trifluoromethane sulfonic acid
MAPS	= monoclonal antibody binding solution (BioRad)
PE	= phycoerythrin
PECy5	= tandem conjugate phycoerythrin and cyanin 5

Description of the Figures:

Fig. 1 Reactivity of mab 7F11 with different WGA purified MUC1 mucins. Antigen was purified by WGA-Sepharose chromatography as described in Example 1 and subjected to 7.5% SDS-PAGE and Western blotting (500 U in 20 µl). T47-D supernatant (track 1), T47-D cytosol (track 2), P1.1 ascites (track 3), P1.2 pleural effusion (track 4), P1.3 ascites (track 5), P1.4 ascites (track 6), P1.5 ascites (track 7), P1.6 serum (track 8), P1.6 ascites (track 9), P1.7 ascites (track 10), P1.8 ascites (track 11) (P: Patient).
ST, standard molecular weight in kilodalton.
Detection of highly glycosylated and underglycosylated MUC1 fractions. Double bands of 400-440 kD in lanes 1, 2, 5 and 7. Underglycosylated MUC1 fractions in the range of 200-220 kDa in lanes 2, 5, 6, 8, 9, 10 and 11.

Fig. 2 Patient No. 1: 7F11 pharmacokinetics and humoral immunity, breast cancer T1b, N0, M0.

Fig. 3 Patient No. 2: 7F11 pharmacokinetics and humoral immunity, breast cancer T1b, N1b, M0.

Fig. 4 7F11 pharmacokinetics. First, second and third immunization.

Fig. 5 HAMA development in patients immunized with 50, 20 and 2.5 mg 7F11.

Table 1

Anti-carbohydrate mabs submitted to the TD4-MUC1 workshop

Clone	TD-4[1]	Isotype	Milk[2]	ZR75[3]	LS[4]	Peptide[5]
27.1	137	IgG1	-	-	-	-
5F4	151	IgM	-	-	+	-
SH1	127	IgG3	+	-	+	-
DH-1	128	IgM	+	-	+	-
HH6	157	IgG3	+	-	-	+
M26	143	IgM	-	-	+	-
43	163	IgM	+	-	+	-
CC2	164	IgM	+	-	+	-
7539MR	176	IgG2	+	+	-	-
BW835	134	IgG1	+	+	-	+
3E1.2	145	IgM	+	+	-	+
LU-BCRJ-G7	173	IgM	-	+	-	+
115D8	148	IgG2b	+	+	-	-
KC4	15-	IgG3	+	+	-	-
Ma695	154	IgG1	+	+	-	-
FH6	167	IgM	-	+	-	-
Antibodies of the invention						
7F11	-	IgG1	-	+	-	-
1E4	-	IgG1	-	+	-	-

1 = antibodies submitted to the TD4-MUC1 workshop

2 = binding with normal human milk MUC1

3 = binding with ZR75 breast cancer cell line MUC1 positive

4 = binding with LS 174T colon cancer cell line MUC1 negative

5 = binding with various synthetic peptides of MUC1

Example 1

Identification and purification of antibodies

Monoclonal antibody 1E4:

Clone 1E4 (IgG1,k) was selected from a BALB/c mouse immunized sequentially for 8 times every 4-6 weeks with MUC1 fractions from three patients. Spleenocytes were fused with X63-P3 X 63Ag8.653 (ATCC CRL1580) mouse myeloma cells according to established methods (1) using PEG 6000. Hybridomas were selected in ten 96-well plates using mouse macrophages as feeder cells. Supernatants were screened by ELISA and immunocytology. 19 hybridoma clones with strong MUC1 reactivity were identified and kryopreserved. Main selection criteria for the clone 1E4 were strong reactivity with various tumor-associated antigen fractions, no reactivity with the immunodominant MUC1-VNTR peptide sequence APDTR (SEQ ID NO:5) und minimal reacticity with normal milk MUC1, deglycosylated MUC1 and normal urinary mucin (polymorphic urinary mucin-like glycoprotein PUM).

Monoclonal antibody 7F11:

Clone 7F11 (IgG1,k) was derived after 8 immunizations of a BALB/c mouse with MUC1 fractions purified from ascites of two patients and the breast cancer cell linie T47-D. After fusion of spleenocytes with P3X63Ag8.653 myeloma cells nine hybridomas with MUC1 specificity were identified. Selection criteria were the same as for 1E4, namely strong reactivity with secreted MUC1 but minimal reactivity with normal milk MUC1, deglycosylated MUC1 and PUM.

Methods

1. Cells

Mycoplasm free myeloma P3X63Ag8.653 was used. The murine Ig nonproducer cell line was grown in Dulbeccos modification of Eagles medium (DMEM) with 4.5 g/l (D+) glucose, 0.7 g/l $NaHCO_3$, 10% fetal calf serum (FCS), 15 mM HEPES, 0.5% penicillin-streptomycin and 2 mM glutamine (DMEM, all Gibco, Eggenstein, DE).

Breast cancer cell lines T47D (HTB133), SKBR3 (HTB30), MCF7 (HTB22), ZR75 (CRL1500) were received from the ATCC. KS, WA, AR, KM22 and HG15 breast cancer cell lines were established from ascites (KS), primary carcinomas (WA, AR) and bone marrow (KM22 and HG15 of breast cancer patients). Mucin negative control cell lines SW1116 (CCL233) and LS174T (CCL188) are from the ATCC. Human fibroblasts were isolated from connective tissue

of breast cancer patients. Ovarian carcinoma cell lines HI and OC were established from ascitic fluids.

2. Purification of MUC1 glycoprotein fractions from ascitic fluids and pleural effusions
Ascites and pleural effusions from 26 patients with advanced breast cancer were collected, cleared by membrane filtration and analysed for MUC1 content by EIA. Highly positive samples of 2 to 3 liters from individual patients were frozen at -20°C and used for antigen purification.

3. Production of standard antigen (BMA) from breast cancer cell lines KS and T47-D
T47-D cells (malignant pleural effusion, ductal breast carcinoma, ATCC HTB133) and the cell line KS, were grown in Nunc factories (Nunc, Wiesbaden, DE). Batches of 2 x 10^9 cells were collected by trypsination and washed twice with PBS. Cells were homogenized in 500 ml lysis buffer (10 mM TRIS/HCl pH7.6, 2 mM EDTA, 1% Triton® X-100, 1% aprotinin and 0.1 mM PMSF) for 1 h at 4°C under constant agitation. Nuclei were removed by centrifugation at 10,000 g for 15 min and the supernatant (cytosol) was stored at -70°C or used for antigen purification.

4. Purification of MUC1
4.1 Antigen purification for hybridoma production
4.1.2 Lectin affinity chromatography
Cytosol, ascites or pleural effusions were purified by lectin affinity chromatography on Wheat germ agglutinin (WGA) Sepharose (Pharmacia, Freiburg, DE) in 2x30cm columns at a flow rate of 2-5 ml/min. Columns were washed with low and high salt (2 M NaCl) PBS and glycoproteins were eluted with N-acetyl-glucosamin (125 mg/ml) at a flow rate of 1 ml/min, dialysed against PBS and concentrated 10-fold by ultracentrifugation with YM100 membranes (Amicon, Weiden, DE). Protein content was analysed using a micro assay according to Lowry (BioRad, München, DE).

4.1.3. Gel exclusion chromatography
Lectin affinity purified fractions of ascites and cytosol proteins were purified by Superose 6 fast protein liquid chromatography (FPLC, Pharmacia) in 2.5 x 100 cm columns. Samples of 5 ml were applied and separated at a flow rate of 4 ml/min in PBS, 2 mM EDTA. MUC1 positive fractions in the molecular weight range of 200 to 1,000 kDa were analysed by EIA and adjusted to 2-10 µg/ml and used for immunization of BALB/c-mice and for specificity tests of hybridoma supernatants. Antigen was stored at -20°.

4.2. Purification of MUC1 by immune affinity chromatography

The mab 2E11, reactive with native und deglycosylated MUC1, was coupled to Affi-Gel 10 (BioRad) at a concentration of 10mg antibody per ml gel. Cytosol was applied in batches of 250 ml to the antibody column at a flow rate of 1 ml/min followed by extensive washing with PBS and PBS, 2 M NaCl. Bound antigen was eluted with 7M urea, pH 6, at a flow rate of 0.5 ml/min and dialysed overnight against 5 l PBS. Purified antigen was then passed through a 5 ml goat anti-mouse IgG-Agarose (Sigma, Deisenhofen, DE) column to remove trace amounts of 2E11. Protein was analysed according to Lowry (BioRad) and adjusted to 200 µg protein/ml. This breast mucin antigen (BMA) can be used as standard in MUC1 assays at a dilution of 1:35 - 1:100 for the 500 U standard value.

5. SDS-PAGE and Western immunoblotting

Proteins were separated under reducing conditions in 7.5 % acrylamide gels (16x16 cm Protean, BioRad), according to Laemmli and transferred to nitrocellulose at 300 mA for 4 h in glycin/methanol buffer. Nitrocellulose was blocked with PBS, 01% Tween 20 and 2% dry milk powder for 1 h. Purified antigen fractions and deglycosylated antigens of different sources were probed with with biotinylated mabs (1 µg/ml PBS, 1% BSA) for 1 h and detected by strepavidin peroxidase and 4-chloro-1-naphthol as substrate.

6. Enzymatic and chemical deglycosylation of MUC1

Affinitiy-purified MUC1 samples were dialysed against 0.1 M PP, pH 5.5 (neuraminidase) or 0.1 M PP pH6.0 (O-glycanase) and incubated at 37°C for 24 h with neuraminidase (2, 5, 10, 20 mU, Vibrio cholerae, Boehringer Mannheim GmbH, Mannheim, DE) or O-glycanase (2, 20 and 200 mU, Diplococcus pneumonie, Boehringer), respectively. Digestion was stopped by heating samples for 5 min to 100°C.

Chemical deglycosylation with TFMSA was performed as described by Edge, A.S.B., et al., Anal. Biochem. 118 (1981) 131-137). Purified antigen (0.1 mg) was lyophilized and incubated for 1 and 3 h at 4°C with 200 µl TFMSA, cooled to -80°C, neutralized with 50% aqueous pyridine and dialized against 0.1% ammonium bicarbonate.

7. Development of MUC1 peptide specific mab 2E11

T47-D cytosol proteins were partially purified by Superose 6 gel exclusion chromatography. The high molecular weight fraction of 1-2 x 10^6 Da was used to immmunize BALB/c-mice according to established methods. Final immunizations were performed with neuraminidase and O-glycanase treated antigen. Sleenocytes were fused with X63Ag853 myeloma cells. Hybridoma 2E11 was selected by ELISA, cytology and histology. The mab (IgG3,k) showed strong and broad reactivity with formalin-resistant epitopes of breast and other carcinomas. The antibody could be used alone and in combination with 7F11 and 1E4 for quantitative determination of serum MUC1 levels. A two site sandwich EIA tests with 7F11 as coating ab and peroxidase labeled 2E11 was developed for MUC1 quantification. Mab 2E11 was cloned 11 times by limiting dilution.

8. Immunization and selection of clone 1E4

MUC1 was purified by WGA-chromatography and Superose-6 gelfiltration from ascites of patients FO, OC and ST. Female BALB/c mice were immunized sequentially for 8 times every 4-6 weeks with antigens from diferent patients. Spleenocytes were removed 4 days after a booster immunization and fused with X63-Ag853 mouse myeloma cells according to established methods (Kaul, S., Entwicklung monoklonaler Antikörper mit Spezifität für Mammakarzinomzellen, Habilitationsarbeit, Zentrum der Biologischen Chemie der Universität Frankfurt, 1983) using PEG 6000. Hybridomas were selected in ten 96-well plates using mouse macrophages (PM) as feeder cells. Supernatants were screened two weeks later by ELISA and cytology. 19 hybridoma clones with strong MUC1 reactivity were identified and kryopreserved. Primary selection criteria for the clone 1E4 were minimal reacticity with normal milk MUC1,

deglycosylated MUC1 and normal urinary mucin (polymorphic urinary mucin-like glycoprotein PUM), strong cytological reactivity with breast cancer cell lines T47D and KS and minimal cross reactivity with human fibroblasts, respectively. 1E4 was cloned 8 times by limiting dilution.

9. Immunization and selection of clone 7F11

MUC1 was purified by WGA-chromatography and Superose-6 gelfiltration from ascites of patients ST, ZE and from the breast cancer cell line T47-D. BALB/c-mice were immunized sequentially for 8 times with the different antigen fractions. After fusion of spleenocytes with X63-Ag853 myeloma cells nine hybridomas with MUC1 specificity were identified. Clone 7F11 (IgG1,k) was selected by ELISA and cytology and was characterized by minimal reactivity with normal milk MUC1, deglycosylated MUC1 and PUM and strong reactivity with MUC1 from serum, ascites and tumor cells. Mab 7F11 was cloned 12 times by limiting dilution.

10. Immunization and selection of clone 5A6

MUC1 was purified by WGA-chromatography and Superose-6 gelfiltration from serum of patient ZI and from secreted MUC1 (tissue culture supernatant) of T47-D cells. Both antigen fractions were mixed and used for three immunizations of BALB/c-mice. After fusion (Feb. 1990) of spleenocytes with X63-Ag853 myeloma cells 24 hybridomas with MUC1 specificity were identified. Clone 5A6 (IgG1,k) was selected by ELISA and cytology using native and deglycoylated antigen fractions from serum, ascites, tumor cells and normal cells. 5A6 was cloned 10 times by limiting dilution.

11. Hybridoma cloning

For the selection of stable high-producer clones, hybridoma cells were seeded at densities of 0.5, 1, 2 and 5 cells well in DMEM with 10% FCS, 50 U penicillin/ml, 50 µg streptomycin/ml in 96-well plates containing $1x10^4$ PM as feeder cells. Six days later plates were inspected under the microscope to identify single hybridoma clones. Clones were tested for specific antibody production by EIA in antigen coated plates. 6-8 high producer clones from every cloning step were grown up to bulk cultures and frozen in liquid nitrogen.

All clones were finally adapted to growth in antibiotic free DMEM/nutrient mix F12 (Gibco) with 15 mM HEPES, 0.8 g $NaHCO_3$/l, 4.5 g/l glucose, 2.5 % FCS (Gibco, origin USA), supplemented with transferrin, insulin, Selen (Boehringer Mannheim GmbH) and 0.5 % bovine serum albumin (Albumax, Gibco) (complete D/H medium).

The Ig production of 2E11, 1E4, 7F11 and 5A6 in D/H-medium was is in the range of 15-25 µg/ml in stationary cultures.

12. Antibody production

Antibodies were produced in a CellPharmII unit (Unisyn, Heraeus, DE) using hollow fiber bioreactors type 3570 (70 kDa membrane). $2x10^9$ hybridoma cells were inoculated in complete D/H-medium with 2.5% FCS. Serum was reduced within 3 weks to 1%. During the production phase hybridomas were grown at a medium feed rate of 4 liters/day. Every day 100-200 ml supernatant was harvested, analysed for Ig content, cleared by membrane filtration and stored at -20C. There was no decline in the Ig synthesis rate during two to three months production cycles.

13. Antibody purification

Mabs were purified by Protein A-Sepharose 4 Fast Flow (Pharmacia). Bioreactor supernatants were mixed with an equal volume of binding buffer, pH8.9 (MAPS, Bio-Rad) and batches containing of 250 mg mouse Ig were applied on 20 ml protein-A gel at a flow rate of 1 ml/h (FPLC-system Pharmacia). IG was eluted with 0.1 M citrate buffer, ph 3.9, neutralized with 2M HEPES, pH 9.0 and dialysed extensively against either PBS or 0.1 M NaHCO3, pH 8.4. Purity was analysed by Superdex 200 (Pharmacia) gelfiltration and SDS-PAGE using 15% acrylamid gels (Ready Gels, Bio-Rad) and silver staining (Bio-Rad). Monoclonal antibodies were sterilized by membrane filtration and stored at concentrations of 1, 2 and 5 mg/ml at -20°C.

14. Labeling of mabs

For cytology, histology and EIA the antibodies were labeled (5 mg, 2 mg/ml in 0.1 M $NaHCO_3$, pH 8.4) with biotin (long arm NHS-Biotin, Vector, Burlingame, USA) and peroxidase (Boehringer Mannheim GmbH, DE) according to the instructions of the manufacturer. Labeling of mabs with FITC, PE and PECy5 for FACS analysis was performed by Cymbus (Dianova, Hamburg, DE).

15. MUC1 quantification by two site enzyme immunoassay (EIA)

Maxisorb immuno plates (Nunc) were coated with mucin glycon specific mab 7F11 (500 ng/well) using phosphate buffered saline (PBS, pH7.6) for 1 h at 37°C. Plates were washed with PBS and blocked with PBS,1% bovine serum albumin (BSA) for 1 h and washed 3 times with wash buffer (WB:150 mM NaCl, 50 mM Tris, pH 7.6, 0.05%Tween 20). Test samples and standard antigen (100yl) were added for 30 min at 37°C at apropriate dilutions in PBS,

0.5% BSA, 0.5% TritonX100 (AD, antibody diluent). Plates were washed 4 times with WB and MUC1 was determined using the peptide (APDTR) specific mab 2E11 labeled with peroxidase (2E11-PO, 2 mg/ml 1:40,000 in AD, 30 min, 37°C). Substrate BM-Blue (Boehringer), 100 µl/well, was added for 30 min at 37°C. Standard antigen (BMA, breast mucin antigen) was immune affinity purified from the breast cancer cell lines KS and T47-D.

16. Synthetic MUC1-VNTR peptides

Synthetic peptides of the MUC1 tandem repeat structure were synthesized on a solid peptide synthesizer (Dr. Nastainzyk, Univ. Homburg/Saar) und coated at a concentration of 100ng/well in EIA plates (Maxisorb, Nunc) using 0.1 M carbonate buffer, pH9.4. The peptides M20 (APDTRPAPGSTAPPAHGVTS) SEQ ID NO:1, P24 (APDTRPAPGSTAPPAHGVTSAPDT) SEQ ID NO:2, M5-15 (PAPGSTAPPA) SEQ ID NO:3, and M6-17 (APGSTAPPAHG) SEQ ID NO:4 were used for epitope analysis.

17. Epitope characterization by flow cytometry and competitive ELISA

Binding to KS and T47-D cells (breast carcinoma, MUC1 positive), LS174T (colon carcinoma, MUC1 negative) was analyzed by flow cytometry (Coulter EPICS) after labeling of native or methanol fixed cells with 7F11 and 1E4 directly conjugated with FITC, PE or PE-Cy5. For epitope characterization the antibodies of the invention were competed with the commercially available anti-MUC1 antibodies HMFG1, HMFG2, Ma552, Ma695, b12, DF3, 115D8, BC2, BC3, SM3, VU11E2, VU12E1, KC4 and MF06.

General information about epitopes identified by anti-MUC1 antibodies of the invention was gained by ELISA using different sources of antigen. MUC1 from ascites of breast and ovarian cancer patients, serum from breast cancer patients, human milk, human urine, cytosol fractions from KS, T47-D and LS174T cells were purified according to 4.1.2 and 4.1.3. Maxisorb immunoplates (Nunc) were coated with 0.1 µg antigen per well in carbonate buffer, pH 9.6 for 18 hours at room temperature. After washing with PBS unspecific binding was blocked by incubation with PBS, 1% BSA. Antibodies of the invention were used as biotinylated reagents at concentrations of 5, 10 and 20 ng/well. For competition unlabeled anti-MUC1 antibodies were added in the concentration range of 1 to 100 ng/well. Plates were incubated for 2 hours at 37°C. After washing streptavidin-peroxidase (1.20 000 Dianova, Hamburg, DE) was added for 20 min. at RT. After washing, the OD was determined at 450 nm after incubation for 20 min. with BM Blue POD (Boehringer Mannheim GmbH, DE) substrate.

Results

1. Epitope specificity of 1E4 and 7F11

The epitope specifictity of 1E4 and 7F11 was analysed on a large panel of MUC1 fractions isolated from different normal and tumor sources (Table 2). In contrast to 2E11 and several other well characterized mabs with specificity for the immundominant MUC1 peptide epitope PDTR (HMFG1, HMFG2, b12, DF3) the mabs 1E4 and 7F11 are completely negative with several overlapping peptides containing the PDTR sequence. The antibodies did not react with glycoproteins from normal human urin (PUM) and human milk. The epitopes of both antibodies are insensitive to mild proteolytic treatment (trypsin) and excessive deglycosylation with neuraminidase, however the antibody reactivity is completely abolished by treatment of purified MUC1 with neuraminidase and O-glycanase. Analysis of MUC1 separated by SDS-polyacrylamide gel electrophoresis and Western immunoblotting shows reactivity of both mabs with high (400-440 kDa) and lower molecular weight fractions (180, 200-220 kDa). In addition, 1E4 and 7F11 showed completely different staining profiles with unpurified MUC1 from serum and ascites of breast cancer patients with advanced disease. Generally there is a broad reactivity with antigens in the molecular weight range from 180 - 440 kDa. These data suggest that 1E4 and 7F11 are reactive with epitopes present in underglycosylated MUC1 fractions which are overexpressed and secreted in carcinomas and absent or only fortuitously expressed in MUC1 of normal (epithelial) cells.

Both mabs are characterized by a strong reactivity with secreted antigen in sera, ascites and pleural effusions.

2. Comparative histological analysis of mabs 2E11, 1E4, 7F11 and 5A6

2.1. Normal tissues

The MUC1-specific mabs 7F11, 1E4 as well as the antibodies 1A6 and 2E11 were screened for specificity on a large panel of normal tissues and carcinomas of different organs.Staining was performed with biotinylated mabs (0.5 µg/ml) and streptavidin-alkaline phosphatase with new fuchsin as substrate either manually or using an automated immunostainer (Dako Techmate).

As can be seen from Table 3 normal cells and tissues of skin, peripheral blood, connective and fat tissue, smooth and striated muscle, blood vessels, cartilage, bone, heart, liver, tongue, spleen, adrenal and brain have been negative with all antibodies in all cases. In breast (acini, ducts), colon (enterocyts), endometrium, pancreas (acini, ducts), prostate, placenta, stomach (parietal cells), kidney (distal tubulus, collecting ducts) and lung (ciliated epithelium) MUC1 is detected with peptide specific mab 2E11 in the apical border of secretory cells and in secretion products. Almost all mabs with PDTR peptide specificity (HMFG1, HMFG2, 115D8, DF3,

F36/22, M26, b12; overview cf. Price, M.R., et al., Summary Report on the ISOBM TD-4 Workshop: Analysis of 56 Monoclonal Antibodies against MUC1 Mucin, Tumor Biol. 19, suppl. 1(1998) 1-20) are characterized by this staining pattern. MUC1 expression was also documented with mab 2E11 in erythroblasts and a small fraction of plasma cells of the bone marrow.

In contrast to this, the mabs 7F11 and 1E4 showed a much more restricted reactivity with normal epithelial cells. In the normal breast 7F11 was positive with acini and ducts in 4 from 12 cases, with heterogenous staining in individual cases. 1E4 reacted only occasionally with acini, single epithelial cells and secretion products. In colon, endometrium, lung, pancreas and placenta 7F11 reacted only weakly with the apical membrane of epithelial cells, while 1E4 was completely negativ. The only organ with consistently strong reactivity with 2E11 and 7F11 was the kidney, while 1E4 was weakly positive with ductal membranes in three cases.

2.2. Tumor tissues

The tumor reactivity of mabs 2E11, 5A6, 1E4 and 7F11 is shown in Table 4. Routine immunhistological staining on formalin fixed, paraffin embedded tissues have shown that 2E11 and 7F11 are reactive with over 96% of primary breast carcinomas, while 1E4 is reactive with more than 90% of primary breast carcinomas. In contrast to the homogenous staining pattern of MUC1-peptide specific mabs (2E11, HMFG1) with breast and various other carcinomas, the mabs 7F11, 1E4 and 5F2 are characterized by a more heterogenous staining pattern in individual tumors. Typically in 50% of the cases there are areas of rather weak and very strong expression of the respective epitops in the same tumor. Approximately 40% of the 1E4 positive tumors show focal cytoplasmic staining of the golgi complex, while strong reactivity is found only in small tumor areas, single cells and secretion products.

2.3. Analysis of micrometastatic tumor cells in bone marrow

Comparative analysis of micrometastatic tumor cells in bone marrow was performed on formalin/methanol fixed cytospins ($1x10^6$ cells/slide) using 2E11, 7F11 and 1E4 (0.5 µg/ml) with the APAAP-technique (Dako Techmate). All slides were evaluated with the discovery picture analysis (Becton Dickinson). Control antibodies for tumor cell analysis were CAM5.2 (cytokeratin 8/18) and 5D3 (cytokeratin 8/18/19). 2E11, 1E4 and 7F11 showed strong labeling of tumor cells. While the application of 2E11 was hampered by significant cross reactivity with erythroblasts and plasma cells, 7F11 had only marginal reactivity with erythroblasts, while 1E4 did not show any cross reactivity with normal bone marrow cells (n = 12), normal peripheral

blood lymphocytes (n = 48) and 42 non-carcinoma patients with haematologic diseases. The sensitivity of the standard cytology with mabs 7F11 and 1E4 was in the range of one tumor cell among 10^6 bone marrow cells or PBL. The comparative analysis of micrometastatic tumor cells in bone marrow of 416 patients with primary breast cancer is shown in Table 5. Two cytospins (2×10^6 BM cells) were stained with every mab. Slide evaluation by picture analysis showed comparable sensitivity and specifictiy of mucin specific 7F11, 1E4 and cytokeratin specific 5D3 for the detection and quantification of micrometastatic cells.

Both mucin specific mabs offers new perspectives for the improvement of rare cell detection by immuncytology, ELISA, flow cytometry, immunomagnetic tumor cell enrichment and for the development of effective purging techniques for rare tumor cells in peripheral blood stem cell collections.

Table 2

Epitope specificity of 2E11, 5A6, 1E4 and 7F11: analysis with different MUC1 fractions isolated from normal and tumor tissues, serum, ascites and with the M24 MUC1 peptide

Antigen	2E11	5A6	1E4	7F11
human milk (HMFGM)	3	1	0	1
human normal PUM	3	0	0	0
human fibroblasts Cyt	0	0	0	0
T47D Cyt	3	1	1	2
T47D WGA	2	2	2	2
T47D BMA 500U	3	2	2	2
T47D BMA N	3	2	2	2
T47D BMA N+G	3	1	0	0
P29 BMA	2	2	2	2
LS174-T Cyt	0	0	0	0
SW1222 Cyt	0	0	0	0
K562 (EL)	1	0	0	0
M24 MUC1 peptide	3	1	0	0
FO (A, WGA)	2	nd	3	2
P1.8 (A, WGA)	2	nd	2	2
P1.9 (A, WGA)	2	2	3	3
P1.6 (S, WGA)	2	2	2	3
P1.6 (S, WGA) N+G	2	0	0	0

score: 3 = strong, 2 = medium, 1 = weak, 0 = negative
result of ELISA with MUC1 fractions as coating antigens

WGA: WGA-sepharose purified antigen

BMA: 2E11-immune affinity purified antigen

Cyt.: TritonX100 solubilized cell material

A: ascites

S: serum

P24 (APDTRPAPGSTAPPAHGVTSAPDT)

N: neuraminidase treated BMA

N+G: neuraminidase and O-glycanase treated BMA

EL: erythroleukemia cell line

Table 3

Reactivity of Mabs 2E11, 5A6, 1E4 and 7F11 with normal tissues (formalin fixed paraffin sections, 1 µg/ml mab, APAAP-technique)

Tissue	2E11		5A6		1E4		7F11	
Adrenal	0/6		0/6		0/6		0/6	
Bone marrow	7/12	E, P	0/5		0/12		1/12	E
Brain	0/6		0/6		0/4		0/4	
Breast	12/15	a,s	1/6	s,f	1/6	s, f	2/6	f, s,
Colon	6/10	c,m	0/6		0/6		3/6	m
Endometrium	12/12		0/3		0/3		2/3	m
Heart	0/6		0/6		0/3		0/3	
Lung	6/6	m,	w1/6	m	0/4		4/6	m
Liver	0/14		0/3		0/3		0/3	
Kidney	10/10	m	w6/6	m, s	w3/6	m	6/6	m
Pancreas	6/6	c,s	w1/3	f	1/3	f	w3/3	m
Ovary	0/6		0/6		0/6		0/6	
Lymph node	0/12		0/15		0/10		0/10	
Spleen	0/5		0/5		0/3		0/3	
Prostate	3/3	m	1/3		0/3		0/3	
Placenta	8/8	m	0/3		0/8		4/8	m
Tongue	0/3		0/3		0/2		0/2	
Testis	0/6		0/6		0/3		0/3	
Stomach	6/6		0/6		0/3		1/3	m
Uterus	0/4		0/4		0/4		0/4	
Urinary bladder (urothel)	7/7		w3/5		0/3		6/6	
Thyroid	w2/3	m,s	0/3		0/3		0/3	
Connective tissue	0/15		0/10		0/10		0/10	
Peripheral blood cells	2/60		0/15		0/24		0/65	
Skin: epidermis	0/15		0/10		0/8		0/15	

E: cytoplasmic and membrane staining of erythroid progenitor cells (3-7% of CD34- cells)

P: plasma cells

m: membrane staining (expression is limited to the apical border of secretory cells)

f: focal intracytoplasmic staining (golgi complex)

s: staining of secretory components

w: staining intensity weak

Table 4

Reactivity of mabs 2E11, 5A6, 1E4 and 7F11 with malignant neoplasms (formalin fixed paraffin sections, 1 μg/ml mab, APAAP-technique)

Tissue	2E11		5A6		1E4		7F11	
	no	int.	no	int.	no	int.	no	int.
Breast cancer	205/219	1-3	46/71	1-3 h,f	66/71	h,1-3	288/302	1-3 h
Breast cancer, bm	all pos *	2-3	14/15	2	<95%pos	2	all pos**	2
Ovarian cancer	8/9	1-3	2/9	1-3	3/9	h,1-3	29/32	2-3
Endometrial cancer	6/6	3	2/4	2	4/4	2	4/4	2
Pancreatic cancer	9/10	2	0/4		1/4	f	4/4	2
Lung cancer, adeno	5/5	1-3	2/5	1-3	5/5	1-3	5/5	1-3
s. c. lung cancer,	10/13	2	10/13	2	10/13	2	10/13	2
Stomach	2/4	2	1/4	2	2/4	2	2/4	2
Liver	0/4		0/4		0/4		0/4	
Renal cancer	10/10	1-3	2/10	1-3	10/10	1-3	10/10	1-3
Prostate cancer	8/10	1-2	8/10	1-2	8/10	1-2	8/10	1-2
Prostate cancer, bm.	2/3	2	nd	2	4/6	2	5/6	2
undiff. tumor	0/4		0/4		0/4		0/4	
epith. undiff. tumor	4/4	3	2/4	3	2/4	3	3/4	3
Leiomyoma	0/4		0/4		0/4		0/4	
Mesothelioma	4/6	1-2	0/6		0/6		3/6	1
Basal cell carcinoma	2/4	1	0/4		0/4		0/4	
Sarcoma	2/75		0/5		0/5		0/5	
Lymphoma	18/46	2	0/24		0/24		0/24	
Melanoma	0/8		0/8		0/8		0/8	

score: 3 strong, 2 medium, 1 weak intensity

bm: bone marrow

m: membrane staining, typically **not** limited to the apical border of tumor cells

c: homogenous cytoplasmic staining

f: focal intracytoplasmic staining (golgi complex)

s staining of secretory components

*: 2E11: showed strong (2-3) staining of micrometastatic tumor cells in more than 1000 patients, however there was significant crossreactivity with plasma cells and erythroblasts of normal bone marrow

**: 7F11: strong staining of all micrometastatic tumor cells, with occasional weak staining of erythroblasts

Table 5

Comparative analysis of micrometastatic tumor cells in bone marrow of patients with primary breast cancer

Slide	2E11	1E4	7F11	CAM	5D3
% pos	79.6	46.3	51	44.7	51.3
% neg	20.4	53.7	49	55.3	48.7
n	411	395	412	396	416
neg	84	212	202	219	203

Example 2

Clinical study on immunotherapy of breast cancer patients with minimal residual disease

Patients:

Breast cancer patients with minimal residual disease after standard therapy (Table 6) were included. There was no evidence of clinical metastasis (M0) in 49 patients. Inclusion criterium was documented tumor cell contamination of the bone marrow at the time of the primary operation or/and after standard adjuvant therapy.

Clinical protocol:

After informed consent patients were treated in the department of gynecology and remained under medical supervision for 24 h (50 mg and 20 mg treatment group). Pretreatment consisted of infusion of 2 mg clemastinhydrogenfumarat (Tavegil) and oral application of 500 mg Calcium. Patients then received 50 mg 7F11 (1 mg/ml) diluted in isotonic NaCl i.v. over a period of 8 hours. The 20 mg dose was diluted in 500 ml NaCl and infused over 4 hours. These patients could leave the hospital one hour later. Patients of the 2.5 mg had no premedication and received the antibody in 250 ml NaCl by a one hour infusion and could leave the hospital one hour later.

Before treatment venous blood samples were collected and analysed for a) immune status, tumor markers (CA153, CEA, BM27), HAMA and anti-7F11. In selected patients 7F11

pharmakokinetics was analysed in the serum 4, 12, 48 and 72 h after antibody application. During infusion blood pressure was monitored every hour.

Venous blood samples were analysed 2 and 4 weeks after each immunization for HAMA, ab2, ab2ß and ab3.

Mononuclear cells from peripheral blood were prepared before immunization, a time points when significant ab2 titers were induced and before and 7 days after booster immunizations. Cells were frozen in liquid nitrogen for future phenotyping, B-cell and T-cell culture and FACS analysis.

Detection of micrometastatic tumor cells in bone marrow:
Bone marrow samples were separated by Ficoll densitity centrifugation and mononuclear cells were used for the preparation of cytospin slides with $1x10^6$ bone marrow cells per slide. Cells were air dried and fixed with 3.5% neutral buffered formalin for 15 min., washed with PBS and then fixed for another 5 min. with cold (-20C°) 100% methanol. Slides were washed with PBS, transfered to storage buffer (PBS with 60g/l sucrose, 0,4 g/l $MgCl_2$ and 43% glycerol) and stored at -20°C. Epithelial tumor cells were stained after a blocking step for alkaline phosphatase (20% acetic acid for 10 min., 2.3% $NaJO_4$ for 10 min.) and normal serum (5% normal mouse serum for biotinylated primary antibodies, 5% human serum for the APAAP technique) by immunocytology using biotinylated 7F11 (0.5 µg/ml), 5D3-biotin (Novocastra, 1:100) and MOC31 (anti-epithelial specific antigen, Biogenex, 1:300). Slides were proccessed using the Dako Techmate 500 immunostainer with either streptavidin-alkaline phosphatase (Dako), goat-anti mouse IgG alkaline phosphatase $F(ab)_2$ (Coulter, 1:250) or the APAAP system (Dako) and new fuchsin as substrate. Tumor cells were evaluated and quantitated by the Discovery image analysis system (Becton Dickinson). Tumor cell analysis of patients immunized with 7F11 was controled by additional double staining techniques using alkaline phosphatase kits with Vector Red (biotinylated primary antibody) and Vector Blue (APAAP technique) (Vector, Burlingame, USA) to exclude staining of B-cells and plasma cells with isotype (mouse IgG1 and 7F11) specificity.

Results from at least 6 slides were expressed as tumor cells per 10^6 normal bone marrow cells (TCD).

Analysis of humoral immune response:

HAMA were assayed with ELISA by coating with mouse IgG. Sera were diluted twofold from 1:20 to 1:1000 and binding was assayed by peroxidase labeled mouse IgG according to the instructions of the manufacturer (Medac, Hamburg, DE).

Anti-7F11-antibodies:

Patient sera were adsorbed to mouse IgG-Agarose for 18 h and preadsorbed sera were added to ELISA plates coated with 7F11-F(ab)$_2$-fragments. Human ab2-antibodies were determined with peroxidase labeled 7F11. Standard antigen was prepared from a serum pool of patients showing low HAMA but high 7F11 titers. 7F11 was coupled to AffiGel 10 (BioRad) at a concentration of 5 mg/ml according to the instructions of the manufacturer. Human serum with high anti-7F11 titers was diluted 1:2 with binding buffer, pH 8.9, and applied to the 7F11 gel at a flow rate of 0.2 ml/min. Human anti-7F11 Ig was eluted with 0.1 M citrate buffer, pH 3.0, dialysed against PBS and used as standard antigen in the concentration range of 500 to 2.5 ng/ml. For ab2ß analysis the IgG fraction was affinity purified using mouse IgG agarose.

Competition ELISA for ab2ß determination:

Mouse IgG preadsorbed sera were incubated at concentrations of 0.1 to 10 µg/ml in 7F11-F(ab)$_2$ coated microtiter plates. For competition the plates were incubated 18 hours with purified MUC1 antigen in the concentration range 500 units/ml. Human ab2ß were determined using goat anti-human IgG labeled with peroxidase.

Human anti-MUC1 reactivity:

ELISA plates were coated with native MUC1 purified by WGA-Sepharose chromatography from the breast cancer cell lines KS and T47-D. Control antigen were cytosol fractions from MUC1 negative SW1116 colon carcinoma cells. Synthetic MUC1 peptides of the VNTR (p24) were coated at a concentration of 1 µg/ml. Sera collected before and two weeks after every immunization were screened for anti-MUC1 reactivity. Sera were added at a dilution of 1:25 for 1 h at 37°C. Human IgG and IgM was identified with class specific goat anti-human antibodies labeled with peroxidase (Dianova, Hamburg, DE) and BM-Blue as substrate.

Immune phenotyping:

Activation of lymphocytes during therapy was analysed using a panel of CD markers by 4 color flow cytometry (Coulter Epics).

Clinical aspects

Patients:

55 patients with breast cancer were immunized repeatedly with murine mAb 7F11. The number of tumor cells (TCD) in bone marrow before and after therapy and the number of immunization and the dosage of 7F11 is shown in Table 6. The mean TCD for all patients is 3.2 tumor cells/ 10^6 mononuclear bone marrow cells before therapy. Clinical data of the tumor stage, type and pretreatment of the study group are presented in Table 2. Staging of the tumor was performed according to the TNM classification. 27 patients were lymph node negative, while 28 were node positive. Adjuvant chemotherapy preceded the 7F11 therapy in 32 cases, while 18 patients continued standard hormone therapy during the 7F11 treatment.

7F11-Immunization:

Initially the 50 mg treatment dose was infused over 120 minutes. Five patients of this 50 mg treatment group had mild immediate allergic reactions (exanthema, hypotension, fever). All symptoms lasted < 24 hours and resolved spontaneously without specific therapic. Patient Nr. 8 developed symptoms of exanthema, low fever, hyoptension and brochospasm. The side effects were completely reversible by infusion of only 8 mg dexamethason (Fortecortin). Later the 500mg treatment dose was infused over eight hours. Under these conditions allergic side effects could be avoided, even in patients which had developed allergic symptoms during the first immunizations. No patient developed long lasting toxic side effects. To date, seven patients have received maintenance booster immunizations of 50 mg 7F11 without any side effects. All 12 patients of the 20 mg dosage group (infusion time 4 hours) and all 21 patients of the 2.5 mg treatment group (infusion time 1 hour) showed no adverse drug effects.

Pharmacokinetics of 7F11:

The pharmacokinetics of native murine mAb 7F11 in serum is shown in Table 7. Patients receiving 50 mg 7F11 had serum peak concentrations in the range of 6-18 µg/ml 4 hours post infusion. There was a rather slow decline to 8 µg/ml after 12

hours and 6 µg/ml after 48 hours. The peak serum concentrations of 7F11 decreased in parallel with rising HAMA and human anti-7F11 titers.

HAMA and ab2 antibodies:

At present 18 patients of the 50 mg group and 6 patients of the 50/20 mg group have finished the immunization. The HAMA analysis in these treatment groups showed great variation in time and titer.Slightly elevated levels before treatment were recorded in three patients. Under treatment one group of patients is characterized by a very slow increase and by a low steady state of HAMA titers, not exceeding 1µg/ml (patients 3, 6, 11, 13, 14 and 20). Most patients however showed a continuous rise in HAMA levels resulting in titers of more than 10 µg/ml after 4 to 6 immunizations.

In contrast to this, the anti-7F11 titers raised significantly only after the second immunization. During threapy all patients of the M0 group developed stable titers in the range of 2-8 µg/ml during further immunizations. Typical HAMA and anti-7F11 titers of individual patients during 7F11-therapy are presented in Figs. 2 and 3. HAMA and anti-7F11 titers in the treatment groups with 20 mg and 2.5 mg antibody are not significantly different from that of the 50 mg group.

Generally HAMA and 7F11 titers showed the same course during immunization in the majority of the patients. After therapy serum titers showed a slow declined with a half life time of 4 to 6 months. Booster immunizations after one year (7 patients) led to dramatic increases and sustained HAMA and anti-7F11-titers.

Inhibition assays with purified mucin as competitor in selected patients (No. 1, 2, 4 and 5) clearly showed that a significant fraction of the ab2-antibodies was specific for the variable region of the therapeutic mAb 7F11 (ab1) and therefore were ab2ß antibodies.

Analysis and follow up of the surrogate marker micrometastatic tumor cells (TCD) in bone marrow:

Nineteen patients of the M0 group have completed the immunotherapeutic trial. Comparison of TCD before (mean value 3.3 tumor cells) and after therapy is shown in Table 6. Two patients (no 1and 3) were analysed twice after therapy. The other patients were analysed at time points indicated in the Table. In each case at least 6 cytopsin preparations with 6 x 10^6 bone marrow cells were stained with antibodies against cytokeratin (5D3) and MUC1 (7F11, 1E4) and human epithel-specific antigen (MOC31). Cases which showed isotype reactivity were analysed by additional double stainings. The bone marrow aspirations of 18 patients of the M0 group were scored negative after 7F11-therapy, while one patient (No. 29) with significant anti-mouse reactivity was scored positive (71 10^6) and will be reanalyzed in future.

Eight patients with documented metastasis were included in the 7F11 immunization protocol. Three patients with elevated serum levels of the tumor markers CA153 (pat. no. 34), CEA (pat. no. 27) and Ca125 (pat. no 16) before therapy showed a remarkebly slow and delayed HAMA- and anti-7F11 titer development (Table 7).

To date six patients of this group could be analyzed for TCD during and after immunotherapy. Elimination of tumor cells in bone marrow was seen in two cases (No. 12, local disease, and No. 17), a no change was recorded in another patient (No. 10, local disease). In a third patient (No. 16) with documented bone metastases and elevated tumor marker levels of CA125 before therapy a steady increase of TCD was recorded.

Example 3

3.1 Treatment scheme for 2.5 mg dosage

Patients with positive bone marrow findings are immunized 6 to 8 times at four-weeks-intervals. Aim: stable HAMA titer.

Blood withdrawals for laboratory tests:

prior to each 7F11 treatment

1) blood count
2) 1 white monovette for testing the patient-associated tumor markers
3) 1 white monovette for determining HAMA, anti-7F11
4) 1 blue monovette for determining the immune status

Pretreatment:

None

7F11 application:

The antibody 7F11 is present at a concentration of 1 mg/ml in physiological saline. For intravenous application 2.5 ml of antibody solution are diluted in 250 ml of physiological saline. Infusion is performed for over one hour. Blood pressure is checked every hour. After a follow-up period of one hour, patient can leave the clinic.

Last immunization:

Each patient receives on the day of the last immunization an envelope containing a monovette (white) for a further blood withdrawal four weeks after immunization. Arrangement of date for clinical control examination. Fixing a date for the bone marrow control examination three months after the last immunization.

3.2 Treatment scheme for 20 mg dosage

Patients with positive bone marrow findings are immunized 6 to 8 times at four-weeks-intervals. Aim: stable HAMA titer.

Blood withdrawals for laboratory tests:

prior to each 7F11 treatment

1) blood count
2) 1 white monovette for testing the patient-associated tumor markers
3) 1 white monovette for determining HAMA, anti-7F11
4) 1 blue monovette for determining the immune status

Pretreatment:

1) 2 tablets calcium
2) short infusion with Tavegil: 1 ampoule of 2 mg in 5 ml in 100 ml NaCl

7F11 application:

The antibody 7F11 is present at a concentration of 1 mg/ml in physiological saline. For intravenous application 20 ml of antibody solution are diluted in 500 ml of physiological saline. Infusion is performed for four hours. Blood pressure is checked every hour. After a follow-up period of one hour, patient can leave the clinic.

Last immunization:

Each patient receives on the day of the last immunization an envelope containing a monovette (white) for a further blood withdrawal four weeks after immunization. Arrangement of date for clinical control examination. Fixing a date for the bone marrow control examination three months after the last immunization.

Example 4

Evaluation of the acute effect

In an examination of the acute-toxological effect of the MUC1 antibody, 2.28 mg of the antibody, in 1.2 ml PBS, corresponding to 9.5 mg/kg, were injected intravenously into 12 female Sprague Dawley rats (supplied by Charles River Wiga; age: 8-9 weeks, weight: 231 $\pm$ 9 g). This dosage corresponds to about 25 times the dosage intended for female patients (0.38 mg/kg = 25 mg per 65 kg body weight).

Despite this foreign application no changes in behavior and no clinical signs of intolerance, and, in particular, no symptoms of an allergic reaction (shock, change in rate of breathing) were observed during the i.v. injection of this mouse antibody. The animals' behavior after awakening from the narcosis (3-4 minutes after injection, anaesthetic: N_2O, O_2, halothane) was, without exception, completely unremarkable.

From this it follows that the mouse antibody BM-7, when administered in the dose specified above, shows no acute effect on the cardiovascular system in rats.

Table 6

7F11 Immunotherapy of breast cancer patients with minimal residual disease - overview 1/98

Patient No	start of therapy	mg BM7	no of imm.	M	TCD before	TCD
1.	31.07.95	50	5	0	1	neg. 10/95
2.	31.07.95	50	10	0	1	neg. 9/96
3.	30.08.95	50	10	0	14	neg. 8/96
4.	04.09.95	50	6	0	1	neg. 2/96 neg. 2/97
5.	12.09.95	50	10	0	4	neg. 6/96
6.	10.11.95	50	8	0	1	neg. 7/96
7.	21.02.96	50	6	0	5	neg. 6/96
8.	07.03.96	25	7	0	1	neg. 10/96
9.	21.03.96	50	6	0	1	neg. 11/96
11.	23.04.96	50	10	0	3	neg. 7/97
13.	29.05.96	50	7	0	1	11/97 neg. 11/97
14.	10.06.96	50	6	0	1	neg. 2/97
15.	02.08.96	50	6	0	6	neg. 2/97
18.	20.12.96	50	6	0	3	neg. 11/97
19.	15.01.97	50/20	9	0	-	
20.	20.01.97	20	10	0	6	
21.	04.02.97	50/20	7	0	2	
22.	05.02.97	50/20	7	0	3	neg 11/97
24.	19.02.97	20	6	0	1	neg 11/97
25.	21.02.97	50/20	5	0	2	
26.	26.02.97	20	9	0	2	
28.	07.04.97	20	8	0	2	

29.	14.04.97	20	7	0	3	7/10^6 1/98
30.	23.04.97	20	6	0	2	

(Table 6 continued)

Patient No	start of therapy	mg BM7	no of imm.	M	TCD before	TCD
31	25.04.97	20	5	0	1	neg. 1/98
32	05.06.97	2,5	7	0	2	
33	06.06.97	2,5	6	0	1	
35	27.06.97	2,5	6	0	2	
36	27.06.97	2,5	6	0	1	neg 11/97
37	03.07.97	2,5	6	0	7	
38	23.07.97	2,5	5	0	5	
39	24.07.97	2,5	5	0	4	
40	24.07.97	2,5	5	0	1	
41	29.07.97	2,5	5	0	7	
42.	12.09.97	20	3	0	2	
43.	16.09.97	2,5	3	0	4	
44.	16.09.97	2,5	3	0	1	
45.	23.09.97	2,5	3	0	2	
46.	30.09.97	2,5	3	0	1	
47.	17.10.97	2,5	2	0	1	
48.	29.10.97	2,5	2	0	2	
49.	29.10.97	2,5	2	0	1	
50.	04.11.97	2,5	2	0	3	
52.	17.11.97	2,5	1	0	4	
53.	20.11.97	2,5	1	0	2	
54.	17.11.97	2,5	1	0	2	
55.	05.12.97	2,5	1	0		

Table 7

7F11 Immunotherapy of breast cancer patients with metastatic disease - overview 1/98

Patient No.	start of therapy	mg BM7	no of imm.	M	TCD before	TCD
10.	01.04.96	50	16	LR*	2	$2/10^6$ 11/96 $1/10^6$ 7/97
12.	23.05.96	50	8	LR*	6	$4/10^6$ 9/96 neg. 9/97
16.	09.09.96	50	10	met.	16	$10/10^6$ 12/96 $45/10^6$ 7/97
17.	26.09.96	50	7	met.	7	neg. 12/97
23.	09.02.97	50	3	met.	>1000	>1000
27.	02.04.97	20	7	met.	3	$1/10^6$ 9/97
34	26.07.97	20	6	met.	1	
51	05.11.97	2,5	2	LR*	1	

M: metastasis

LR: local recurrence

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PATENT CLAIMS

1. An immunologically active polypeptide which specifically binds to the carbohydrate structure of the MUC1 tandem repeat from carcinoma cells, wherein
   a) the quotient between the affinity of the said polypeptide for a 200 to 440 kDa glycoprotein fraction from tumor cell-containing ascites of breast cancer patients and for native MUC1 antigen (400 to 440 kDa) from normal cells is 100 : 1 or more (molecular weight ranges analyzed by SDS gel electrophoresis),
   b) the polypeptide does not bind to nonglycosylated MUC1 antigen, and
   c) the binding of the polypeptide to the said 200 to 440 kDa glycoprotein fraction changes by 10% or less if the glycoprotein fraction was treated with neuraminidase to cleave N-terminal neuraminic acids, or with formalin.

2. An immunologically active polypeptide as claimed in claim 1, wherein it is obtainable from the cell lines DSM ATCC 2328 or DSM ATCC 2329.

3. A method for the production of antibodies which specifically bind to a hypoglycosylated MUC1 fraction from tumor cells, particularly carcinoma cells with a molecular weight of 200 to 440 kDa, wherein the glycoproteins are isolated from tumor cell-containing body fluid, particularly carcinoma cell-containing body fluid. . of carcinoma patients by lectin affinity chromatography, said glycoproteins are optionally separated from the Ig proteins, and the fraction so obtained is used for the immunization of animals, antiserum is obtained, and antibodies are isolated therefrom.

4. Method as claimed in claim 3, wherein said antibodies being characterized in that

   a) the quotient between the affinity of the said polypeptide for a 200 to 440 kDa glycoprotein fraction from tumor cell-containing, particularly carcinoma cells ascites of carcinoma patients and for native MUC1 antigen (420 to 440 kDa) from normal cells is 100 : 1 or more,
   b) the polypeptide does not bind to nonglycosylated MUC1 antigen, and
   c) the binding of the polypeptide to the said 200 to 440 kDa glycoprotein fraction changes by 10% or less if the glycoprotein fraction was treated with neuraminidase to cleave N-terminal neuraminic acids, or with formalin.

5. Method as claimed in claim 3 or 4 for use with breast cancer patients.

6. A method for the production of the immunologically active polypeptide of one of the above mentioned claims, wherein said polypeptide is produced by an expression of a recombinant nucleic acid coding for said polypeptide in prokaryotic or eukaryotic host cells, and subsequently isolating the desired polypeptide from the host cell or the supernatant.

7. Antibodies for the inhibition of proliferation of tumor cells, particularly carcinoma cells in a patient, wherein a pharmaceutically effective amount of an antibody as claimed in one of the above mentioned claims is applied to the patient.

8. A method for the production of a pharmaceutical composition for the inhibition of proliferation of tumor cells, particularly carcinoma cells in a patient, wherein the pharmaceutical composition contains a pharmaceutically effective amount

of an antibody as claimed in one of the above mentioned claims as an essential component.

9. Antibodies as claimed in claim 7 or 8, wherein the antibody is applied one to ten times at weekly or monthly intervals

10. Antibodies as claimed in claims 7 to 9, wherein an amount of 2 μg to 20 mg/kg body weight of the antibody is applied in each case.

11. Antibodies as claimed in claims 7 to 10, wherein the antibody is applied in such a way that the titer of said antibody in body fluids of the patient is kept at a stable level over a period of about three months.

12. Antibodies as claimed in one of the above mentioned claim for the removal of tumor cells, particularly carcinoma cells or secreted MUC1 antigen from a patient's body material , wherein the body material is brought into contact with an immobilized antibody of said claim, the tumor cells or said secreted MUC1 antigen are bound to the immobilized antibody, and the tumor cells or said secreted MUC1 antigen so bound to the solid phase are separated along with the solid phase from the body material.

13. A method for influencing the activity of a tumor patient's T cells, wherein an antibody as claimed in one of the above mentioned claims is applied to the patient.

14. A conjugate consisting of a polypeptide according to claim 1 or 2 covalently bound to a toxin or a cytotoxic substance.

15. A method for the determination of tumor cells, particularly carcinoma cells by incubating samples from a patient with a polypeptide according to claim 1 or 2, wherein there is measured the binding of the antibody to MUC1.

16. A composition for the determination of MUC1 in body fluids, containing a polypeptide according to claim 1 or 2.

17. A method for the histological differentiation of normal cells and tumor cells, particularly carcinoma cells wherein the binding of a polypeptide according to claims 1 and 2 to the cells is investigated.

Fig. 1

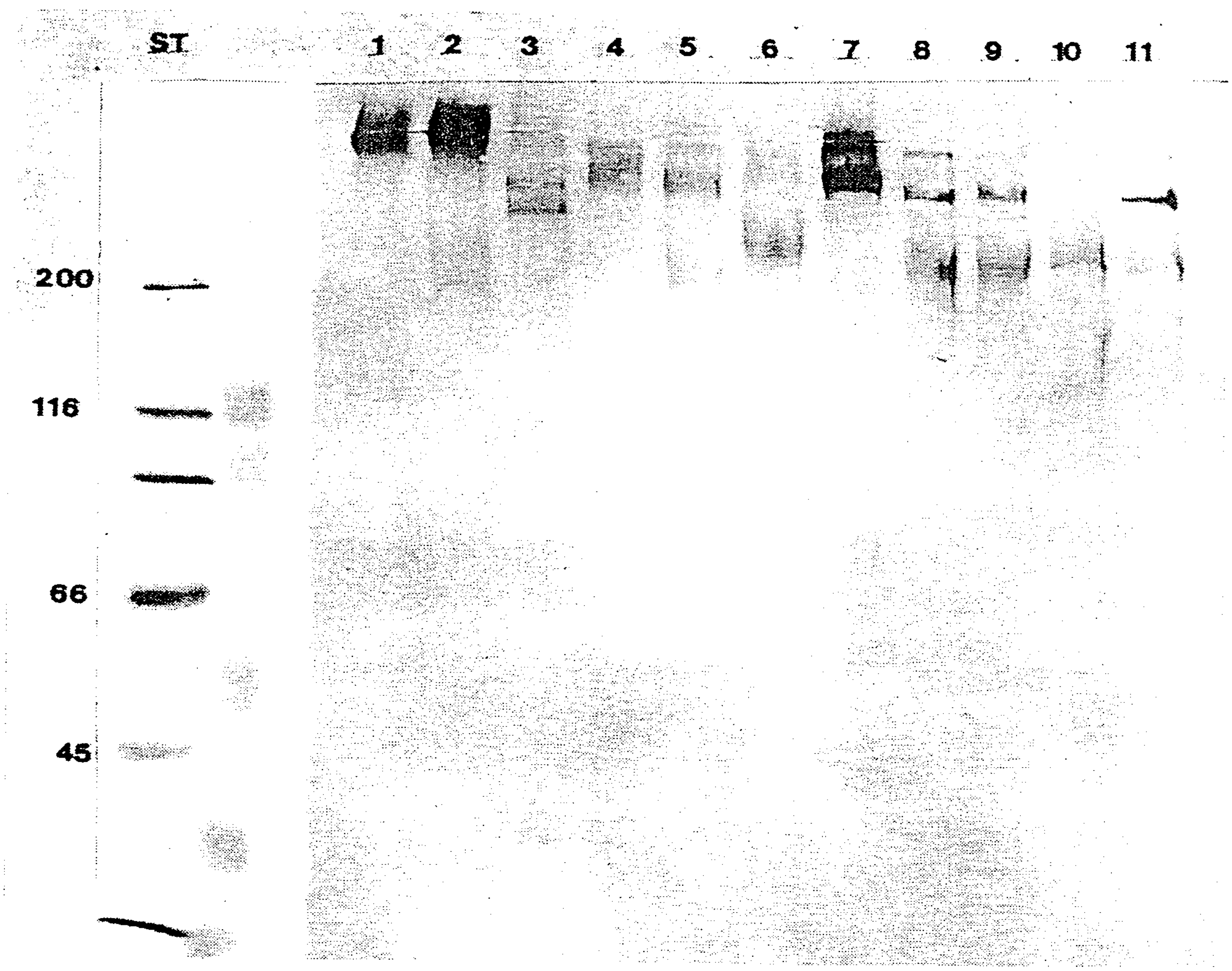
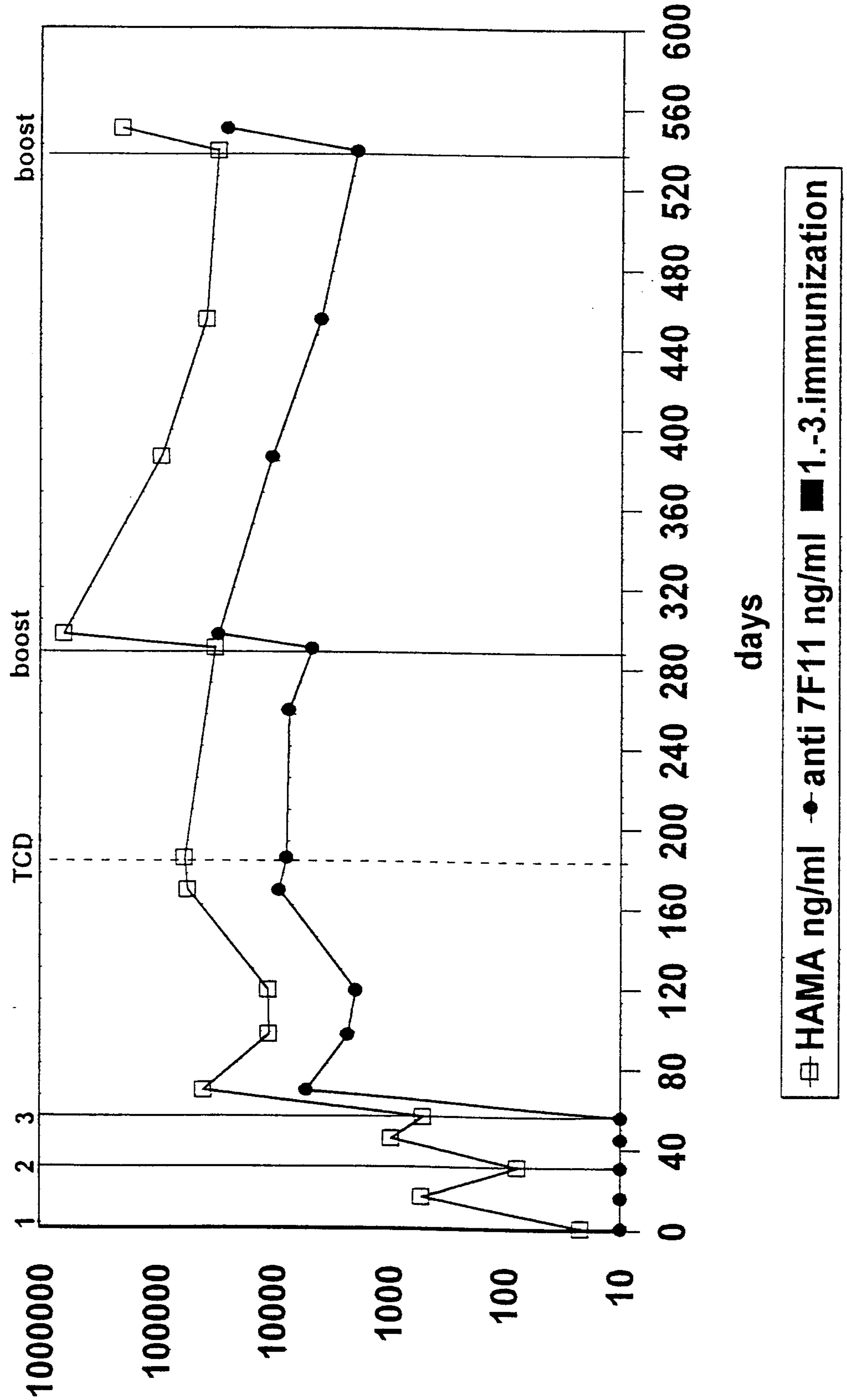


Fig. 2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520
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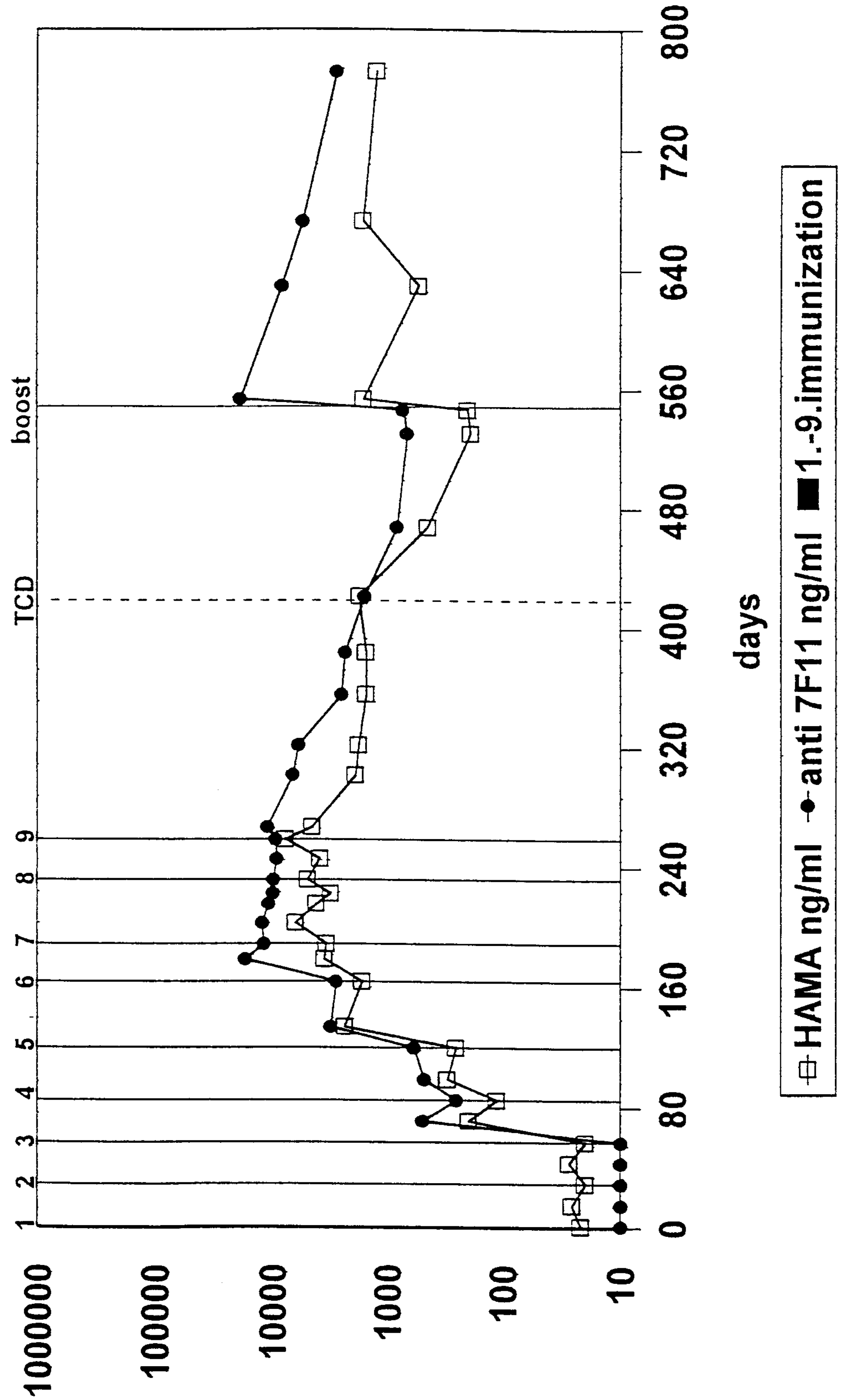
days
HAMA ng/ml
anti 7F11 ng/ml
1.-3.immunization

Fig. 3



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days
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1.-9.immunization

Fig. 4

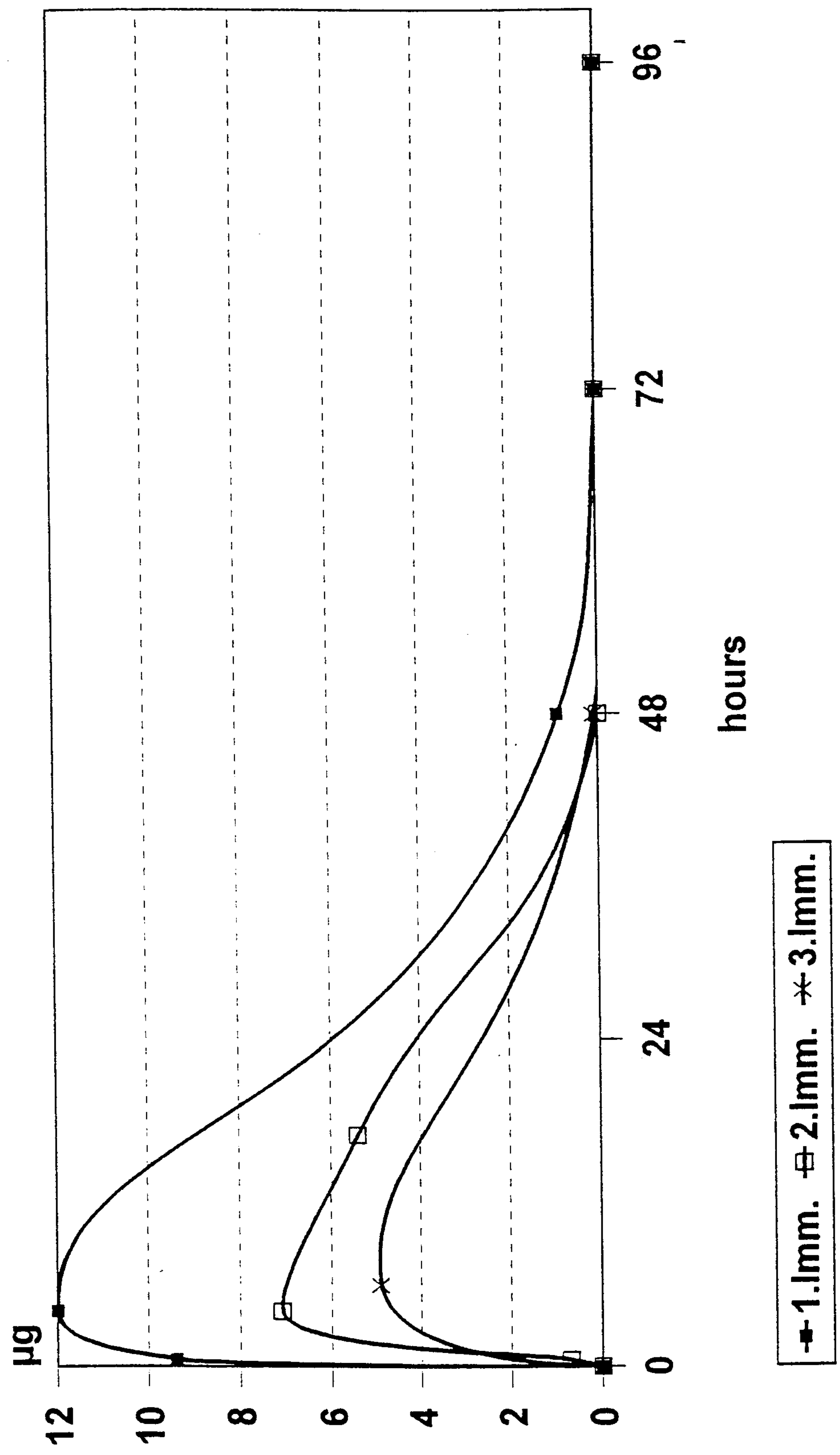
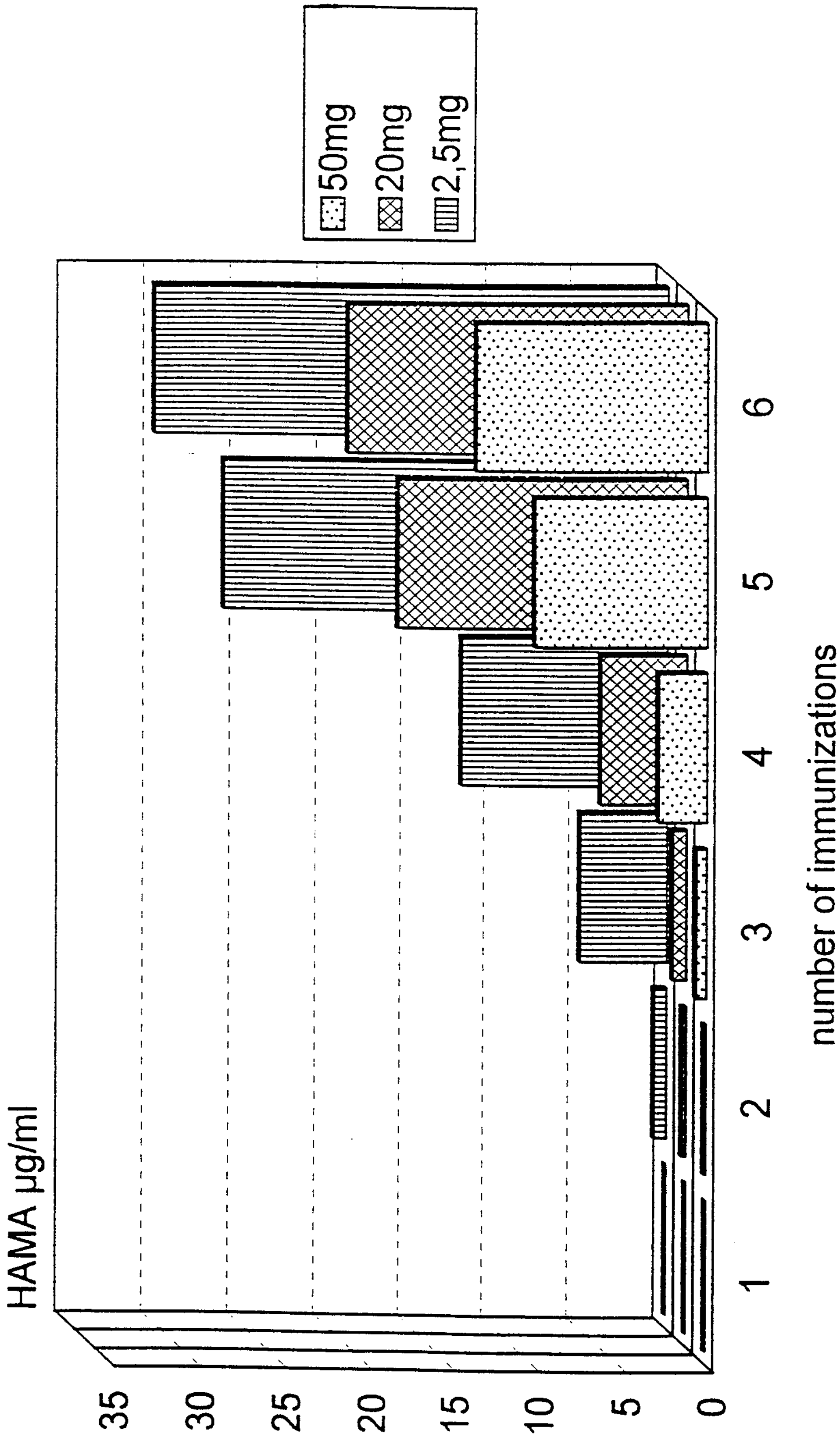


Fig. 5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30
25
20
15
10
5
0
1
2
3
4
5
6
number of immunizations
50mg
20mg
2,5mg

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT :

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(F) POSTAL CODE (ZIP): D-69115

(ii) TITLE OF INVENTION: Specific antibodies against mammary tumor-associated mucin, method for production and use

(iii) NUMBER OF SEQUENCES: 5

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: WINDOWS NT 40

(D) SOFTWARE: MICROSOFT WORD 7

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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Gly Val Thr Ser
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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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Gly Val Thr Ser Ala Pro Asp Thr
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(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

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(2) INFORMATION FOR SEQ ID NO: 5:

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(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

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(54) Title: SPECIFIC ANTIBODIES AGAINST MAMMARY TUMOR-ASSOCIATED MUCIN, METHOD FOR PRODUCTION AND USE

(57) Abstract

An immunologically active polypeptide which specifically binds to the carbohydrate structure of the MUC1 tandem repeat from carcinoma cells, wherein a) the quotient between the affinity of the said polypeptide for a 200 to 440 kDa glycoprotein fraction from tumor cell-containing ascites of breast cancer patients and for native MUC1 antigen (400 to 440 kDa) from normal cells is 100:1 or more, b) the polypeptide does not bind to nonglycosylated MUC1 antigen, and c) the binding of the polypeptide to the said 200 to 440 kDa glycoprotein fraction changes by 10 % or less if the glycoprotein fraction was treated with neuraminidase to cleave N-terminal neuraminic acids, or with formalin. The immunologically active polypeptide, e.g. antibody, is useful in the diagnosis and therapy of breast cancer.

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 99/00941

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K39/395 C07K16/30 C12P21/08 G01N33/574 G01N33/577

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	AGRAWAL ET AL.: "Cancer-associated MUC1 mucin inhibits human T-cell proliferation, which is reversible by IL-2" NATURE MEDICINE, vol. 4, no. 1, January 1998 (1998-01), pages 43-49, XP002071241 the whole document ---	1-17
Y	CIBOROWSKI ET AL.: "Screening of anti-MUC1 antibodies for reactivity with native (ascites) and recombinant (bacoluvirus) MUC1 and for blocking MUC1 specific cytotoxic T-lymphocytes" TUMOUR BIOLOGY, vol. 19, no. 1, 1998, page 147-151 XP002071242 the whole document ---	1-17

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Date of the actual completion of the international search	Date of mailing of the international search report
6 October 1999	15. 10. 99
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Muller-Thomalla, K

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JEROME ET AL.: "A survivor of breast cancer with immunity to MUC-1 mucin, and lactational mastitis" CANCER IMMUNOL IMMUNOTHER, vol. 43, no. 6, January 1997 (1997-01), pages 355-360, XP002071243 the whole document ---	1-17
Y	TAYLOR-PAPADIMITRIOU J.: "Report on the first international workshop on carcinoma-associated mucins" INT. J. CANCER, vol. 49, 1991, page 1-5 XP002071244 cited in the application the whole document ---	1-17
Y	PRICE ET AL.: "Summary Report on the ISOBM TD-4 Workshop: Analysis of 56 monoclonal antibodies against the MUC1 Mucin" TUMOUR BIOLOGY, vol. 19, no. 1, 1998, pages 1-20, XP002071245 cited in the application the whole document ---	1-17
Y	LLOYD ET AL.: "Analysis of the carbohydrate specificity of ISOBM TD-4 Workshop anti-MUC1 antibodies" TUMOUR BIOLOGY, vol. 19, no. 1, 1998, pages 118-121, XP002071246 cited in the application the whole document ---	1-17
Y	APOSTOLOPOULOS ET AL.: "Cellular Mucins: Targets for immunotherapy" CRITICAL REVIEWS IN IMMUNOLOGY, vol. 14, no. 3/4, 1994, pages 293-309, XP002071247 cited in the application the whole document -----	1-17

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 99/00941

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

Although claims 3-5 are directed to a method involving a treatment step (immunization) practised on the human/animal body and although claim 13 is directed to a method of treatment of the human/animal body, and although claim 17 is directed to a diagnostic method practised on the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.